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PRINCIPAL INVESTIGATOR: Shu-chun Teng, Ph.D.

CONTRACTING ORGANIZATION: Princeton University
Princeton, New Jersey 08544-0036

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6. AUTHOR(S)
Shu-chun Teng, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Princeton University
Princeton, New Jersey 08544-0036

E-MAIL:
steng@molbio.princeton.edu

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Table of Contents

(4) Introduction

Telomere length is tightly regulated in *Saccharomyces*. This lengthening is dependent on *TLC1*, which encodes the RNA component of telomerase but independent of *RAD52*, which encodes a protein required for most recombination events in mitotic yeast cells. Most cells lacking yeast telomerase die but survivors arise by *RAD52* dependent events: the majority of these survivors have tandem duplications of the sub-telomeric Y' element. However, as presented in previous annual reports, about 10% of the survivors have a different telomeric structure, very long tracts of $C_{1-3}A/TG_{1-3}$ DNA, with some telomeres being as much as 12 kb longer than telomeres in wild type cells. This pattern of exceptionally long telomeres is similar to that seen in human cell lines and tumors that maintain telomeric DNA without expressing telomerase. The maintenance of these long telomeres requires the continuous presence of Rad52p. However, even in the presence of Rad52p, these long telomeres steadily shorten, suggesting that even in cells that maintain their telomeres by telomere-telomere recombination, recombinational mediated telomere lengthening is relatively rare. *PIF1* is a non-essential *Saccharomyces* gene that encodes a 5' to 3' DNA helicases. Mutations in *PIF1* affect telomeres in three ways. First, telomeres in *pif1* cells are longer than in wild type cells. Second, *de novo* telomere addition on spontaneous or induced double strand breaks occurs much more frequently in *pif1* compared to wild type cells. Third, in *pif1* mutants, new telomeres are often added to sequences with very little resemblance to telomeric DNA whereas wild type cells show a strong preference for forming telomeres at sites with stretches of telomere-like DNA. The *pif1* mutants also exhibit increased loss and decreased recombination of mitochondrial DNA and thus have a high fraction of respiratory deficient (petite) cells. In this study we demonstrate that *rad50 tlc1* and *rad51 tlc1* strains exhibit very different growth patterns, and demonstrate that their survivors display unique and distinctive recombination patterns. These findings suggest that the Rad50 and Rad51 proteins play important but different roles in survivor formation in telomerase-minus *S cerevisiae*. We also found that *PIF1* is the founding member of a helicase sub-family, conserved from yeasts to humans. We describe five *PIF1*-like genes, including a Pif1p-like gene from humans.

(5) Body

The linear eukaryotic chromosome was a significant evolutionary advance in the storage of genetic information, but it also presented an inherent problem to conventional DNA replication. In each round of DNA replication, the removal of the RNA primer located at the very end of a given chromosome leaves a small gap that cannot be repaired by conventional DNA polymerases. The free 5' end that these enzymes require for transcriptional initiation is not present at these gap sites and, as a result, a little piece of DNA is lost with each successive round of replication. In *Saccharomyces cerevisiae*, the DNA loss eventually triggers a cell cycle arrest or, in some cells, a bypass of this checkpoint that results in the loss of the entire chromosome and cell death (Sandell and Zakian 1993).

To help solve this end replication problem, eukaryotes contain telomeres, specialized nucleoprotein structures found at the ends of chromosomes. The DNA component of this structure is typically composed of tandem repeats whose length and sequence vary according to the organism. In *S. cerevisiae*, the telomeric DNA consists of about 350 ± 75 base pairs of the heterogeneous double-stranded sequence $C_{1\text{-}3}A/TG_{1\text{-}3}$ running away from the centromere, and ends in a short single-stranded $TG_{1\text{-}3}$ tail (Shampay *et al.* 1984; Wellinger *et al.* 1993a; Wellinger *et al.* 1993b).

Immediately internal to these telomeric sequences lie two subtelomeric elements called X and Y' elements. The X element is present on all telomeres, existing as a variable-length DNA tract that ranges from 0.3 to 3.75 kb (Chan and Tye 1983). More recent studies suggest that the X element is comprised of a number of smaller repeated sequences, some of which are always present, and others which are only found on a subset of the telomeres (Louis *et al.* 1994). In contrast, the Y' element is only found on about two-thirds of the telomeres within a cell and, when it is present, can only assume a single configuration out of a fixed number of possibilities. A 6.7 kb tract (Y'-long) and its 5.2 kb deletion-derivative (Y'-short) represent the two building blocks, and any given Y' element consists of a tandem array of one to four copies of a single type of Y' (Chan and Tye 1983; Louis and Haber 1990; Louis and Haber 1992). Additionally, it has been determined that telomeric chromatin organization assumes a distinctly non-nucleosomal structure known as the telosome (Wright *et al.* 1992). The terminal $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats are part of the telosome, while the X and Y' elements are bound in nucleosomes (Wright *et al.* 1992).

The telomere itself is normally maintained by the enzyme telomerase, which contains an RNA template component and a catalytic reverse transcriptase subunit (Greider and Blackburn 1987). Telomerase uses its RNA template to extend the $TG_{1\text{-}3}$ strand, thereby allowing conventional DNA replication machinery to fill in the complementary $C_{1\text{-}3}A$ strand. The RNA primer at the end of this newly synthesized strand is then excised to yield the short single-stranded $TG_{1\text{-}3}$ tail (Figure 1; reprinted with permission from (Zakian 1995)). It has been determined that the RNA template of telomerase in *S. cerevisiae* is encoded by *TLC1* (Singer and Gottschling 1994), and that the reverse transcriptase subunit is encoded by *EST2* (Lingner *et al.* 1997). In addition, it is known that the genes *EST1*, *EST3*, and *EST4* all play essential roles in telomerase activity, as the deletion of any of the *EST* genes results in a phenotype identical to that of *TLC1* deletion, which consists of a gradual shortening of the telomere and cessation of division after about 50 to 100 generations (Lundblad and Szostak 1989; Singer and Gottschling 1994; Lendvay *et al.* 1996; Lingner *et al.* 1997). Plates of these telomerase-minus cells also assume a distinctive appearance, a slow "senescence" that reflects cell death (Lundblad and Szostak 1989). However, in late cultures, survivor colonies have been observed to appear among the senescing cells (Lundblad and Blackburn 1993; Teng and Zakian 1999).

It has been determined as presented in previous annual reports that these survivor colonies maintain their telomeres via an alternate *RAD52*-dependent recombination pathway (Lundblad and Blackburn 1993; Teng and Zakian 1999). The survivors can also be categorized into two groups based on the substrates for recombination; type I survivors result from amplification of the Y' element, while type II survivors arise from amplification of the terminal $TG_{1\text{-}3}$ tract (Lundblad and Blackburn 1993; Teng and Zakian 1999). These survivors can be distinguished from each other via their restriction fragment patterns after either *Xho*I-digestion (Lundblad and Blackburn 1993) or digestion by *Alu*I, *Hae*III, *Hinf*I, and *Msp*I (Teng and Zakian 1999). Furthermore, it was discovered that type I survivors generated from *tlc1* cells have the ability to spontaneously convert to a type II

pattern during outgrowth, while type II survivors retained their pattern for ~250 cell divisions (Teng and Zakian 1999).

The type II recombination pattern seen in *S. cerevisiae* is of particular interest to researchers because of an apparent link between it and certain types of human cancers. Hanahan and Weinberg (1996) re-defined malignant growth in terms of six essential cellular alterations, one of which is the acquired capability of limitless replicative potential. Normal mammalian cells have an intrinsic limit to cellular division, and cells past this limit senesce, or stop growing (reviewed in Hayflick 1997). This limit is imposed by the inactivity of telomerase, which results in telomeres that shorten with each division cycle (reviewed in Hanahan and Weinberg 1996). In 85-90% of malignant cells, however, the telomere is maintained through an up-regulation of the telomerase enzyme (Shay and Bacchetti 1997; Bryan and Cech 1999). However, a small subset of these cells has been shown to maintain their telomeres through another mechanism termed ALT, which appears to be a type of recombination pathway (Bryan *et al.* 1995; Bryan *et al.* 1997). Indeed, these telomerase-negative cells are distinguished by their exceptionally long and heterogeneous telomeres (Bryan *et al.* 1995), a distinctive phenotype shared by type II survivors in telomerase-minus *S. cerevisiae* (Teng and Zakian 1999).

DNA recombination has been examined extensively in yeast, and it has been determined that *RAD52* is the only gene required for all homologous recombination events (reviewed in Paques and Haber 1999). *RAD52* has also been found to form an epistasis group with the genes *RAD50*, *RAD51*, *RAD53*, *RAD54*, *RAD55*, *RAD56*, *RAD57*, *MRE11*, and *XRS2* (reviewed in Paques and Haber 1999). Among these, *RAD51*, *RAD54*, *RAD55*, and *RAD57* share common phenotypes, and Rad51p has been shown to be a homologue of the RecA protein in *Escherichia coli* (Aboussekhra *et al.* 1992; Shinohara *et al.* 1992). Rad51p binds to both single-and double-stranded DNA (Ogawa *et al.* 1993; Baumann *et al.* 1996), and catalyzes an ATP-dependent strand exchange between a single-stranded circular molecule and a linear duplex (Sung 1994; Namsaraev and Berg 1997). The yeast single-strand binding protein complex RPA inhibits Rad51p-binding to ssDNA (Sung 1997), but enhances the strand exchange reaction (Sung 1994; Baumann and West 1997). The deletion of *RAD51* does not produce nearly the same effect as a *RAD52*-deletion, however, as a *rad51* mutant only exhibits a 4-fold reduction in the rate of spontaneous heteroallelic recombination whereas a *rad52* mutant causes a 3,000-fold reduction (Rattray and Symington 1994).

RAD50, *MRE11*, and *XRS2* form another family within the *RAD52* epistasis group whose proteins share common deletion phenotypes (reviewed in Paques and Haber 1999). It has been determined that these three proteins function as a heterotrimeric complex (Johzuka and Ogawa 1995; Usui *et al.* 1998), and that they participate in a variety of DNA repair and maintenance processes (reviewed in Haber 1998). This protein complex possesses a 5'-to-3' exonuclease activity and appears to be involved in the 5'-to-3' resection of double-strand breaks, processes which are severely hampered by the deletion of any one component (Sugawara and Haber 1992; Ivanov *et al.* 1994; Tsubouchi and Ogawa 1998; Lee *et al.* 1998). However, the deletion of *RAD50* exerts very modest effects on the rates of homologous recombination, and actually increases spontaneous heteroallelic recombination 7- to 10- fold (Gottlieb *et al.* 1989; Ivanov *et al.* 1992).

Evidence of two separate *RAD52*-dependent mechanisms of telomere maintenance has been found in a recent study that employs the deletions of different genes within the *RAD52* epistasis group in a *tlc1* background strain (Le *et al.* 1999). The study investigated the DNA recombination mutants *rad50*, *rad51*, *rad52*, *rad54*, *rad57*, *xrs2*, and *mre11*, and found that survivors were generated in each of the double mutants of recombination genes and *tlc1* except *rad52 tlc1*. It was also noted that the *rad51*, *rad52*, *rad54*, and *rad57* mutations in a *tlc1* strain caused extremely rapid senescence and cell death (Le *et al.* 1999). Furthermore, the observation that the *rad50 rad51 tlc1* triple mutant yielded no survivors suggests that *RAD50* and *RAD51* define two distinct *RAD52*-dependent recombination pathways (Le *et al.* 1999).

PIF1 is a non-essential *Saccharomyces* gene that encodes a 5' to 3' DNA helicases. Mutations in *PIF1* affect telomeres in three ways (Schulz and Zakian, 1994). First, telomeres in *pif1* cells are longer than in wild type cells. Second, *de novo* telomere addition on spontaneous or induced double strand breaks occurs much more frequently in *pif1* compared to wild type cells. Third, in *pif1* mutants, new telomeres are often added to sequences with very little resemblance to telomeric DNA whereas wild type cells show a strong preference for forming telomeres at sites with stretches of telomere-like DNA. The *pif1* mutants also exhibit increased loss

and decreased recombination of mitochondrial DNA and thus have a high fraction of respiratory deficient (petite) cells.

In this study we demonstrate that *rad50 tlc1* and *rad51 tlc1* strains exhibit very different growth patterns, and demonstrate that their survivors display unique and distinctive recombination patterns. These findings suggest that the Rad50 and Rad51 proteins play important but different roles in survivor formation in telomerase-minus *S cerevisiae*. We also found that *PIF1* is the founding member of a helicase sub-family, conserved from yeasts to humans. We describe five *PIF1*-like genes, including a second Pif1p-like gene from humans.

Characterization of type II telomere formation in liquid cultures

Telomeres display a heterogeneous and random lengthening phenotype in telomerase-minus yeasts (Lundblad and Blackburn, 1993; Teng and Zakian, 1999). Our previous work has shown that type I survivors are generated in early generations, but type II survivors can easily take over the majority of the survivor population in liquid cultures (Teng and Zakian, 1999). We examined telomere length changes when type II survivors take over a culture. By culturing cells in liquid medium we have discovered that we can preferentially select for type II survivors. This liquid assay system also minimized the character of irregularly random process for survivor formation on solid plates among individual cells. Independent spore colonies were inoculated into liquid YEPD medium and grown for 48 hours. Cultures were repeatedly diluted 1: 10,000 into YEPD medium every 48 hours. DNA from the survivors was isolated at the time of each dilution and was digested with a mixture of *Alu*I, *Hae*III, *Hin*fI, and *Msp*I and probed with $C_{1\text{-}3}A$ as described by Teng and Zakian (1999). These four base-pair cutters recognize different 4-bp sequences and digested regular chromosomal DNAs to an average of 96 bp, but did not digest in telomeres. There are many sites for these enzyme within Y', including sites 358 bp downstream of the 5' end of Y' and 42 bp upstream of the 3 end of Y'. This assay was used to cut most wild-type telomeric fragments to less than 1.1 kb (Teng and Zakian, 1999), type I telomeric fragments to a major band ~250 bp (Teng and Zakian, 1999, and the arrow sign in Figure 1), while leaving long type II telomeres intact. Telomere length dynamics during *tlc1* survivor formation was measured from six independent spores and they all showed similar Southern blot pattern in this assay (two samples are shown in Figure 1). Telomeres became shorter and shorter in the beginning five dilutions. Type I telomeric fragments developed in the early culture were observed as a major band at ~250 bp (the arrow sign in figure). These *tlc1* telomeres reached the shortest length at the fifth dilution. Surprisingly, instead of gradually lengthening, the telomeres suddenly and dramatically lengthened to even more than 12 kb in the next dilution. The heterogeneous type II telomeric fragments in liquid population was reflect on the smear $TG_{1\text{-}3}$ hybridization from the sixth dilution. This extensive telomere lengthening occurs within a very short period of time (between fifth and sixth dilutions) and suggests that telomere lengthening in survivors requires an unusual mechanism that cannot simply entail a regular gene conversion model (Paques and Haber 1999) using existed short chromosomal telomeres as templates.

Dynamic process of telomeres in telomerase-minus survivors.

Our previous data suggests that only critically short telomeres initiate recombination. To further understand the dynamics of telomere process in telomerase-minus cells, we investigated the fate of single telomeres in survivors. A *tlc1* strain was generated with the *URA3* gene marked at the left end of chromosome VII. Type II survivors were generated by continuously dilution in liquid culture (Teng and Zakian, 1999) and type II survivors were repeatedly restreaked up to 24 times on YEPD plates (~600 generations). When we examined the length of this marked telomere by Southern blot analysis (figure 2), we found this telomere was subjected to continuous shortening and lengthening as expected. When telomeres reached a critically short length (restreak 2 and 18), they initiated recombination. In this experiment telomere was lengthened 2.4 kb between restreak 2 and 4. And the 7L telomere in restreak 18 was less than 100 bp, this telomere was elongated to 1.1 kb between restreak 18 and 20. However we didn't observe any telomere lengthening when telomeres were longer than the wild type length. This result suggests that telomere lengthening in type II survivors only occurs when telomeres are critically short.

***rad50 tlc1* and *rad51 tlc1* strains show distinct growth and recombination patterns**

It has been found that Rad52 protein is continuously required for survivor generation and maintenance (Teng and Zakian 1999) and that either Rad51p or Rad50p is required (Le et al, Genetics, 1999). To determine the effects that different members of the *RAD52* epistasis group exert on survivor generation in telomerase-minus cells, haploid *tlc1*, *rad50 tlc1* and *rad51 tlc1* spores from tetrad master plates were streaked onto a YEPD plate to generate the first re-streak. This procedure was repeated every three days until the senescence phenotype appeared. The *rad51 tlc1* strain senesced extremely rapidly when compared to *tlc1* alone, as the senescence phenotype was always present on the first re-streak in this double mutant . Most *rad51 tlc1* cells die and it was hard to obtain survivors. Frequence of survivor formation is lower in *rad51 tlc1* than in *tlc1* or *rad50 tlc1*. These findings are consistent with the study by Le *et al.* (1999) which reported that *rad51 tlc1* mutants accelerate the generation of survivors compared to *tlc1* mutants alone. We also found that the *rad50 tlc1* strain senesced and generated survivors by the fifth re-streak, as compared to around the seventh re-streak for *tlc1* cells .

Survivors from the *tlc1*, *rad50 tlc1* and *rad51 tlc1* strains were generated by re-streaking tiny colonies from plates exhibiting the senescence phenotype. DNAs from the survivors were isolated and digested with the four base-pair cutters and probed with $C_{1\text{-}3}A$ as previously described (Teng and Zakian, 1999). Examination of the recombination patterns of the *rad50 tlc1* and *rad51 tlc1* survivors revealed a pair of striking and consistent phenotypes (Table 1). While 6.5% (6 of 92) of the *tlc1* survivors examined displayed the type II pattern, all 120 *rad50 tlc1* survivors examined displayed the type I survivor pattern, whereas all 120 *rad51 tlc1* survivors exhibited the type II survivor pattern (figure 3). These findings suggest that the Rad50 and Rad51 proteins play important but separate roles in the formation of survivors in the absence of telomerase.

Determination if *rad50 tlc1* survivors maintain their type I profile

Our previous study discovered that type I survivors from a *tlc1* strain could spontaneously convert to a type II recombination pattern (Teng and Zakian 1999). In that study, three type I *tlc1* survivors were re-streaked 10 times on YEPD plates, and it was found that one of the three survivors converted to a type II pattern (Teng and Zakian 1999). We wished to see if type I *rad50 tlc1* survivors were also susceptible to this conversion. In this study, 12 type I *rad50 tlc1* survivors were restreaked 10 times on YEPD plates as previously described (Teng and Zakian, 1999). We found that none of those 12 type I *rad50 tlc1* survivors converted to type II survivors even after 10 restreaks . Since type II survivors were much easier to obtain in liquid culture, we conducted liquid culture assay to confirm the result from solid plate assay. We inoculated *tlc1*, *rad51 tlc1* and *tlc1 rad50* spore clones into liquid YEPD media and allowed them to grow at 30° for three days. The reason to culture for three days was that *rad51 tlc1* culture could not reach stationary phase after two days due to the slow growth phenotype. The cultures were diluted 1:10,000 every three days up to the eighth dilution, and DNAs were isolated from the population of cells in each dilution. The DNA was digested with the four base-pair cutters and probed with $C_{1\text{-}3}A$ as described previously. While *tlc1* and *rad51 tlc1* cultures converted to type II pattern at the forth and third dilution respectively, the recombination patterns of *rad50 tlc1* survivors maintained their type I profile throughout the duration of this assay (Figure 4). This maintained type I pattern in *rad50 tlc1* survivors was also verified by the *Xho*I digestion Southern blot analysis . It was noticed that a portion of telomeres of *rad50 tlc1* survivors extended to the wild type lengths, but could not extend further as normal type II survivors (Figure 3 and 4). These data suggest that Rad50p is required for type II telomere formation. We also observed that *rad51 tlc1* type II survivors appeared earlier than type II *tlc1* survivors did. This probably just reflects that in order to reach stationary phase cells in *rad51 tlc1* culture actually passed more generations than those in *tlc1* culture.

Pif1p is a member of a sub-family of putative helicases.

As one approach to determine the mechanism by which Pif1p, a 5' to 3' DNA helicase, affects telomeres, we searched for Pif1p-like proteins in yeast and other organisms. When the sequence of the 857 amino acid Pif1p was compared to the translated DNA database, several genes encoding *PIF1*-like proteins were identified. One, a second S. *cerevisiae* gene, predicted a 723 amino acid protein. None of the ~58 other genes in yeast that have the 7 motifs characteristic of helicases had any similarity to Pif1p by the criterion of a BLAST search. At the time we began these experiments, there was no function known for the second *Saccharomyces PIF1*-like gene. However, during the course of this work, the gene was shown to be identical to

RRM3 (R. Keil, pers. comm), a gene that represses recombination between ribosomal DNA (rDNA) repeats. The second Pif1-like gene, from the nematode *C. elegans*, predicts a 674 amino acid polypeptide (Yuji Kohara, unpublished). As the sequence of the entire *C. elegans* genome is known, C. *elegans* has only one *PIF1*-like gene. These Pif1p-like protein and used them to isolate a *PIF1*-like gene from the fission yeast *Schizosaccharomyces pombe* that was predicted to encode a 805 amino acid protein. The *S. pombe* gene was called *rph1*$^+$ (*RRM3/PIF1* homologue). By comparing the Pif1p sequence with the translated database of expressed sequence tags, we found one human gene (accession number AA464521) with low levels of similarity (expectation value of 0.015). We extended this sequence 3' to the poly A tail by further searching of the human gene against the EST database (unigene number Hs.112160). The raw sequence was corrected by inspection of the automated sequencing traces, and when this sequence was compared to the protein database, high levels of similarity to the *PIF1* sub-family were found. We extended the sequence deduced from the database to 449 amino acids using rapid amplification of cDNA ends (RACE) (Frohman, 1993) (Figure 6). We were unable to extend this sequence further because after 449 amino acids it was fused to an Alu element as a result of template switching during cDNA synthesis. The deduced human Pif1p-like sequence was 44% similar and 29% identical to the *Saccharomyces* Pif1p over the entire 449 amino acid region. For comparison, the two *Saccharomyces* proteins, Pif1p and Rrm3p, were 52% similar in this region. The human gene was most related to the *C. elegans* Pif1p-like protein (43% identica). The human gene was less similar to the *Saccharomyces PIF1* gene because the human and *C. elegans* genes both lacked an insert near the carboxyl terminus of the proteins that was present in the yeast gene. This insert was a region of low similarity in the Pif1 sub-family, varying in both size and sequence among the various Pif1-like genes.

Discussion

In this study, we revealed several phenotypes for ALT-like type II telomere formation and maintenance. First we found that type II telomeres only elongated when telomeres reached very short length (figure 1). Telomeres are normally protected by the Rap1 complex in the wild-type cells. In telomerase-minus cells, telomeres become shorter and shorter, and much less Rap1 complexes are able to bind there and protect telomeres. Therefore these shortened telomeres become highly recombigenic. Once recombination and elongation occur, these elongated telomeres are once again subjected to telomere shortening in the absence of telomerase.

Second we observed a surprising phenotype: telomere can extend to12 kb or more within a very short of time during telomere lengthening. As shown in figure 2 and the *Xho*I digestion experiment , all telomeres are shorter than 150 bp in the fifth dilution. Suddenly telomere can extend to 12 kb or more in the next dilution. This size of telomere addition could not be obtained from one round of conventional gene conversion given that there is no telomere fragment ~ 12 kb or more in the fifth dilution to serve as the template. We propose two possibilities that might cause this result (figure 5A and 5B). One possibility is that once recombination is initiated, it can continuously recombine using discontinuous template switches (figure 5A). It was suggested that break-induced replication (BIR) pathway might be used for telomere-telomere recombination (Paques and Haber 1999). According to the BIR model, the primer can be extended from the 3' hydroxyl to the end of chromosome after strand invasion. Perhaps in the absence of telomerase the end of chromosome is treated like a double strand break (DSB). This DSB with recombination machinery still associated might easily invade other telomere templates. Second possibility to generate very long telomere within very short time could be explained by a circular telomere ring being caught by the 3' hydroxyl for strand invasion (figure 5B). Telomeric DNA is made up of repeated sequences, and all repeated sequences have the ability to pop out of the chromosome and form an extrachromosomal circular structure randomly. Circle pop out is induced when cell is under stress condition and genome is unstable. If any of these circular telomeres were served as the recipient template for recombination, the telomere could keep being elongated until the polymerase falls off. Both possibilities above can generate very long and heterogeneous telomeres during very brief time.

Based on the different growth curves of *rad50 tlc1* and *rad51 tlc1* cells, and the inability of *rad50 rad51 tlc1* cells to generate survivors, it has been proposed that there are two different pathways governed by the *RAD52* epistasis group for telomere-telomere recombination (Le *et al.* 1999). Our studies on *rad50 tlc1* and *rad51 tlc1* mutants show that two different members of the *RAD52* epistasis group exert different effects on cell

growth patterns and survivor generation. Consistent with the findings of Le *et al.* (1999), we observed that *rad51 tlc1* mutants died extremely rapidly as compared to *tlc1* alone, whereas *rad50 tlc1* mutants survived much longer, on the order of that seen for *tlc1*. It is generally believed that background recombination allows the continued growth of *tlc1* cells before their eventual senescence (Lendvay *et al.* 1996), a notion that is supported by the observation that the *rad52 tlc1* cells that lack homologous recombination die extremely rapidly.

Examination of survivor generation reveals that the *rad50 tlc1* mutant strain generates exclusively type I survivors, whereas the *rad51 tlc1* strain only generates type II (Table 1 and Figure 3). It has been shown that Rad51p is required to open up regions of chromatin that are normally inaccessible to recombination machinery (Sugawara *et al.* 1995). We surmise that the absence of Rad51p leaves the nucleosome-bound Y' region inaccessible to recombination, thereby preventing the generation of type I survivors. However, the non-nucleosomal telomeric tracts are still available as a recombination substrate, which results in the generation of type II survivors. Our previous data showed that type I survivor developed faster than type II survivors. This might explain why in the absence of type I telomere-telomere amplification *rad51 tlc1* mutants died extremely rapidly as compared to *tlc1* alone.

Rad50p has been shown to function in a complex with Mre11p and Xrs2p. It was known this complex has ability to bind to DSB. Other enzymatic functions such as endonuclease, exonuclease and helicase activities have also been demonstrated in vitro (reviewed in Paques and Haber 1999). Perhaps this complex itself or recruited factors might be required to generate the substrates, such as single-stranded primer and/or template, for telomere-telomere recombination (figure 5C). This process would result in a lower energy barrier for $TG_{1\text{-}3}$ strand invasion, and a higher likelihood of type II recombination. The absence of Rad50p inactivates the protein complex and, according to this model, leaves a poor substrate for terminal recombination, thereby resulting in the generation of type I survivors. These models are consistent with finding that *rad50 rad51 tlc1* mutants are unable to generate survivors (Le *et al.* 1999), as the respective pathways leading to type I or type II generation are both blocked by the absence of the Rad50 and Rad51 proteins. Type II survivors maintain their telomeres in a way similar to mammalian ALT cells. We propose that the Rad50-Mre11-Xrs2 complex is also required for the formation of ALT pathway for telomere maintenance in mammalian immortal cells.

MATERIALS AND METHODS

All general yeast procedures were performed as described in Rose *et al.* (1990). Genotypes of haploid spores were determined by replica-plating the tetrad master plates on selective media. Southern blot analysis was performed as described previously (Teng and Zakian, 1999). The *URA3* probe was made from the *Eco*RV-*Nsi*I fragment of the *URA3* gene.

Strain Construction

The diploid *tlc1/TLC1* UT/WT strain was generated by mating the haploid strains STY105 (*MATa ura3-52, trp1, his3-200, lys2-801 amber, ade2-, tlc1::LEU2* + pRS317 *TLC1*)(Teng and Zakian 1999) and YPH500 UT (*MAT□ ura3-52, trp1, his3-200, lys2-801 amber, ade2-,chrVII-URA3*) (Wiley and Zakian 1995). Diploids obtained by replica-plating on selective media were sporulated and tetrad-dissected to obtain the *tlc1* UT strain. The diploid *rad50/RAD50 tlc1/TLC1* strain was generated by mating the haploid strain STY105 and YPH500 *rad50::HIS3*, kindly provided by Bala Balakumaran (Balakumaran *et al.* 2000). Replica-plating on selective media yielded diploids heterozygous for *RAD50* and *TLC1*, and these were sporulated and tetrad-dissected. The diploid *rad51/RAD51 tlc1/TLC1* strain was generated by disrupting the ORF of one copy of *RAD51* in the diploid STY95 strain by PCR amplification of *HIS3* with overhanging *RAD51* ends, followed by transformation into the diploid (PCR sequences available on request). A Southern blot was used to check the transformants, of which two were chosen for sporulation and tetrad-dissection. Spores from both transformants were used to generate survivors.

Survivor Assays

For solid survivor assay, spores on the master tetrad plate were streaked to YEPD to generate the first re-streak. After three days of growth at 30° (~25 generations), the smallest available single colonies were used to generate the next re-streak. When senescence was observed, tiny colonies were streaked and allowed to grow for six days. Survivors were identified by their large size as compared to the background senescing colonies, and were inoculated into YEPD liquid media and allowed to grow for two days at 30° for DNA preparation.

Liquid survivor assay was performed similar to the solid survivor assay. Spores on the master tetrad plate were inoculated into 10 ml YEPD medium. Cultures were diluted repeatedly 1: 10,000 into YEPD medium for every 48 hours for figure 2 and 72 hours for figure 3 experiments. For both solid and liquid survivor assays, DNAs were isolated and digested with *Alu*I, *Hae*III, *Hin*fI, and *Msp*I (Teng and Zakian 1999), and run on an agarose gel. The survival pathway was determined with a $C_{1\text{-}3}A$ probe of the Southern blot analysis.

Human PIF1 cloning

The human *PIF1*-like sequence was assembled using BLASTN to search the est database with the sequence of EST AA464521 as a query. The resulting homologous EST matches were found to be part of the unigene assembly HS.112160. Most of the sequence was of high quality since it was determined multiple times. Those regions that were present in only one read were manually edited by inspecting the sequencing traces and correcting six compression zones: five insertions and one deletion were made. This sequence was extended using rapid amplification of cDNA ends (RACE) (Frohman, 1993) using human fetal thymus Marathon-ready cDNA (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The primers were the flanking primer AP1 (Clontech) and hPIF1-3' primer 1 5'-CTCCAACAATCCAGGGTCATGCCTTGGC-3'). 1.5-4 kb PCR products were gel-purified and re-amplified with flanking primer AP2 (Clontech) and hPIF1-3' primer 2 (5'-GTGGCCTGCACGTCCAGCGGTCA-3'). The 1.5 kb PCR product was gel-purified and cloned into the pCR,TOPO2.1 vector (Invitrogen). In addition to obtaining new sequence, we also verified, and when appropriate, corrected the sequence in the data base. The sequence of the first RACE was extended using human ovary Marathon-ready cDNA as described above except that we used hPIF1-3' primer 2 and hPIF1-3' primer 3 (5'-GTACCTTACCTGGCAGCTGAAGC-3'). DNA sequencing of both strands was performed with the amplitaq FS dye terminator cycle sequencing kit (ABI) and electrophoresis was on an ABI prism 377 DNA sequencer.

Accession numbers: The accession number for *rph1*$^+$, the *S. pombe* gene is AF07944 and for the human gene is AF108138.

(6) Key research accomplishments

- Telomere-telomere recombination is an efficient bypass replication pathway in telomerase-minus cells.
- PIF1, a helicase for telomere replication, is conserved from yeast to human.
- *RAD50* and *RAD51* affect telomere-telomere recombination differently.

(7) Reportable outcomes

Teng and Zakian, Telomere-telomere recombination is an efficient bypass pathway in telomerase-minus *Saccharomuces cerevisiae*. Molecular and Cellular Biology, (1999) 19, 8083.

Zhou, Monson, Teng, Schulz, and Zakian, Pif1p helicase, a catalytic inhibitor of telomerase in yaest. Science, (2000) in press.

(8) Conclusions

In this study, we revealed several phenotypes for ALT-like tumor-like telomere-telomere recombination and telomere maintenance in telomerase-minus cells. First we found that type II telomeres only elongated when telomeres reached very short length. Telomeres are normally protected by the Rap1 complex in the wild-type cells. In telomerase-minus cells, telomeres become shorter and shorter, and much less Rap1 complexes are able to bind there and protect telomeres. Therefore these shortened telomeres become highly recombigenic. Once recombination and elongation occur, these elongated telomeres are once again subjected to telomere shortening in the absence of telomerase. Second we observed a surprising phenotype: telomere can extend to12 kb or more within a very short of time during telomere lengthening. As shown in figure 2 and the *Xho*I digestion experiment , all telomeres are shorter than 150 bp in the fifth dilution. Suddenly telomere can extend to 12 kb or more in the next dilution. This size of telomere addition could not be obtained from one round of conventional gene conversion given that there is no telomere fragment ~ 12 kb or more in the fifth dilution to serve as the template. Based on the different growth curves of *rad50 tlc1* and *rad51 tlc1* cells, and the inability of *rad50 rad51 tlc1* cells to generate survivors, we found two different pathways governed by the Rad50p and

Rad51p. We propose that the Rad50-Mre11-Xrs2 complex is also required for the formation of ALT pathway for telomere maintenance in mammalian immortal cells. Third I cloned the human homolog of PIF1which is required for completing telomere replication.

FIGURE LEGENDS

FIGURE 1: One step telomere amplification for type II telomere formation. Two independent freshly dissected *tlc1* spores were used to inoculate YEPD medium. Cultures were diluted 1: 10,000 every 48 hours for 12 times. DNAs were prepared from each culture. The DNA was digested with *Alu*I, *Hae*III, *Hin*fI, and *Msp*I, subjected to electrophoresis in a 1.2% agarose gel, prepared for Southern analysis, and probed with a *S. cerevisiae* $C_{1-3}A/TG_{1-3}$ telomeric probe. The major band from type I telomeric fragments is indicates by the arrow. The positions of molecular weight markers in kilobase pairs are indicated.

FIGURE 2: Dynamics process of type II telomeres. A *tlc1* strain was marked with the *URA3* gene (Wiley and Zakian 1995) at the chromosome VII-L. A type II survivor was generated and streaked on rich medium and grown to single colonies (~25 cell divisions). Individual colonies were resteaked repeatedly every three days for 24 times on YEPD plates. DNA was prepared from single colonies of every other restreak. The DNAs was digested with *Pst*I, subjected to electrophoresis in a 1% agarose gel, prepared for Southern analysis, and probed with a *URA3* probe. The telomeric *Pst*I fragment of the *URA3* contains 895 bp of the *URA3* gene plus telomeric sequence. The 7 kb band is from the *ura3-52* mutant at its normal chromosome V. The positions of molecular weight markers in kilobase pairs are indicated.

FIGURE 3: All *rad50 tlc1* survivors are type I and *rad51 tlc1* survivors are type II. Survivors were generated by streaking tiny colonies from the re-streak plate. DNA was digested with *Alu*I, *Hae*III, *Hin*fI, and *Msp*I, and subjected to electrophoresis in a 1.2 % agarose gel. Southern blot analysis was performed as in figure 2. 120 survivors from each strain were examined and 26 are shown here.

FIGURE 4: Separate roles of *RAD50* and *RAD51* for telomere-telomere recombination. Fresh dissected *tlc1, tlc1 rad51,* and *tlc1 rad50* spores were used to inoculate YEPD medium. Cultures were 1: 10,000 diluted every 72 hours for 8 times. DNAs were prepared from each culture, digested with *Alu*I, *Hae*III, *Hin*fI, and *Msp*I, subjected to electrophoresis in a 1% agarose gel. Southern blot analysis was performed as in figure 2.

FIGURE 5: (A). Continuous strand transfer using distinct templates. After one round gene conversion, the 3' hydroxyl of the recombination product is re-invaded into the next template before the recombination machinery falls off, so the telomere can continuously recombine using discontinuous templates. **(B). Circular TG_{1-3} ring serving as the template for strand invasion.** A circular telomere ring is pop out from the chromosomal telomere and caught by the 3' hydroxyl primer of the telomere for strand invasion. According to the rolling circle replication model, the telomere can keep being elongated until the polymerase falls off. **(C). Rad50p and Rad51p roles in survivor formation.** See text for details. (The round shaded circle indicates the recombination machinery and the stripped square indicates the Y' element. Donor telomeres for strand invasion are represented as gray boxes and recipient telomeres are represented as black boxes.)

FIGURE 6: Homology of *PIF1* Helicase Sub-family Members. The predicted sequences of the *PIF1*-like proteins were compared using the TBLASTN 2.06 program. The default parameters of the NCBI blast server were used, except that filtering of low complexity regions was not done. The top line for each pair shows the expectation value, a measure of the probability that the match occurred by chance. The number of amino acids of homology shared between the two proteins and percentage identity within the helicase region is shown below. These numbers were obtained by aligning the helicase region using the MacVector 6.0 (Oxford Molecular) implementation of the CLUSTALW program. As determined, an insertion in either of the two proteins counts in the length calculation and reduces the level of identity. The *PIF1*-like proteins, including a fragment of human sequence, were aligned using the MACAW 2.0.5 program. The default parameters were used, including the BLOSUM62 matrix and the segment pair overlap search method. White areas have a mean identity score of 0-37%, grey is 37-67%, and black is 67-100%. The positions of the seven helicase domains are shown above. The *S. pombe* Rph1p is 805 amino acids, the *Saccharomyces* Rrm3p is 723 amino acids, the *Saccharomyces* Pif1p is 857 amino acids, the *C. elegans* sequence is 674 amino acids, the *C. maltosa* partial protein sequence is 365 amino acids, and the human partial protein sequence is 449 amino acids. The peptide region of the 449

amino acids of the known human sequence and the corresponding regions of the other *PIF1*-like proteins were aligned using the MacVector 6.0. Shaded residues are 60% or more identical among all proteins. The positions of helicase domains are shown above the protein sequence.

TABLE1: Survivors from the *tlc1*, *rad51 tlc1* and *rad50 tlc1* strains were generated by re-streaking tiny colonies from plates exhibiting the senescence phenotype. Types of survivors were determined by Southern blot analysis using four base-pair cutters as described (see Materials and Methods). The *rad51 tlc1* and *rad50 tlc1* survivors examined in the survivor assay were each sporulated from two different *rad51/RAD51 tlc1/TLC1* and *rad50/RAD51 tlc1/TLC1* transformants, respectively.

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(10) Appendices

See attachments.

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Shu-Chun Teng

Department of Molecular Biology
Princeton University
Princeton, NJ 08544
TEL: (609)-258-2723
FAX: (609)-258-1701
E-mail: steng@molbio.princeton.edu
http://www.molbio.princeton.edu/zakian/people.html

[redacted]

[redacted]

PERSONAL DATA

PII Redacted

[redacted]

EDUCATION

1991-96	Ph.D., Biochemistry and Molecular Biology, Dec. 1996 Rutgers University, New Jersey, USA
1985-89	B.S., Chemistry, June 1989 National Taiwan University, Taipei, Taiwan

EXPERIENCE

1997-present	Visiting Research Fellow Topic: Characterization of telomere-telomere recombination in telomerase- minus *Saccharomyces cerevisiae.* Group leader: Virginia Zakian Princeton University, Department of Molecular Biology
1998-present	Instructor of Princeton independent research course 1998-99 and 1999-2000 academic years Princeton University, Department of Molecular Biology
1999	Reviewer of Journal *Genes and Development* Manuscript Number: U-7257
1999	Reviewer of Journal *EMBO Journals* Manuscript Number: EMBO/ 1999/ 18350
1998	Reviewer of Journal *Genetics*

Manuscript Number: 7591

1996-1997 Research Associate
Topic: Identification of the protein components of telomerase in yeast
Laboratory: Virginia Zakian
Princeton University, Department of Molecular Biology

1997 Reviewer of Journal *Nucleic Acid Research*
Manuscript Number: 7T/0195

1992-96 Graduate Assistant
Topic: Analysis of retrotransposons in *Crithidia fasciculata* and *Saccharomyces cerevisiae*
Laboratory: Abram Gabriel
Rutgers University, Department of Molecular Biology & Biochemistry

1992-93 Teaching Assistant
Introduction to Molecular Biology, Course 315
Rutgers University, Department of Molecular Biology & Biochemistry

1989-91 Taiwanese Army
Kaoshing Chemical Arsenal, Taiwanese Army, Kaoshing, Taiwan

1988-89 Student Researcher
Topic: Evolution of pituitary hormones of Taiwanese fishes
Laboratory: Tong-Bi Lo and Yea-Sha Chang
Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan

PROFESSIONAL ACTIVITIES

Speaker
1999, Gordon Research Conference, Plasmid and Chromosome Dynamics, Colby-Sawyer College, NH
1999, Cold Spring Harbor meeting, Eukaryotic DNA replication, Cold Spring Harbor, NY,
Regulation of telomere formation in saccharomyces
1996, Mid Atlantic Transposable Element Meeting, National Cancer Institute, Frederick, MD
1996, Mid Atlantic Yeast Meeting, University of Maryland, Baltimore, MD
1994, Mid Atlantic Transposable Element Meeting, National Cancer Institute, Frederick, MD

Poster presentation
1999, Cold Spring Harbor meeting, Eukaryotic DNA replication, Cold Spring Harbor, NY,
Pif1p, a 5' to 3' DNA helicase , inhibits telomerase mediated lengthening of yeast telomeres.
1996, Gordon Research Conference, Molecular Genetics, Salve Regina University, RI
1995, Mid Atlantic Yeast Meeting, Princeton University, Princeton, NJ

Participant
1998, Gordon Research Conference, Nucleic Acids, Salve Regina University, RI
1997, Annual Worcester Foundation-MBL,Symposium on the Nucleus, Marine Biological Laboratory, Woods Hole, MA
1995, Mid Atlantic Transposable Element Meeting, University of Pennsylvania, Philadelphia, PA

1995, Mid Atlantic Transposable Element Meeting, National Institute of Health, Bethesda, MD
1994, Mid Atlantic Transposable Element Meeting, Carnegie Institute, Baltimore, MD
1993, Mid Atlantic Transposable Element Meeting, Johns Hopkins University, Baltimore, MD

HONORS, AWARDS, GRANTS

1997-present	Department of Defense, U.S. Army Breast Cancer Research Grant number: DAMD17-97-1-7242
1991-92	Rutgers Excellent Graduate Fellowship

PUBLICATIONS:

Teng, S.C., Wang, S.X., and Gabriel A. A new non-LTR retrotransposon provides evidence for multiple distinct site-specific elements in *Crithidia fasciculata* miniexon arrays. (1995) *Nucleic Acid Research*, 23, 15, 2929-2936.

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List of personnel (not salaries) receiving pay from the research effort

Not applicable.

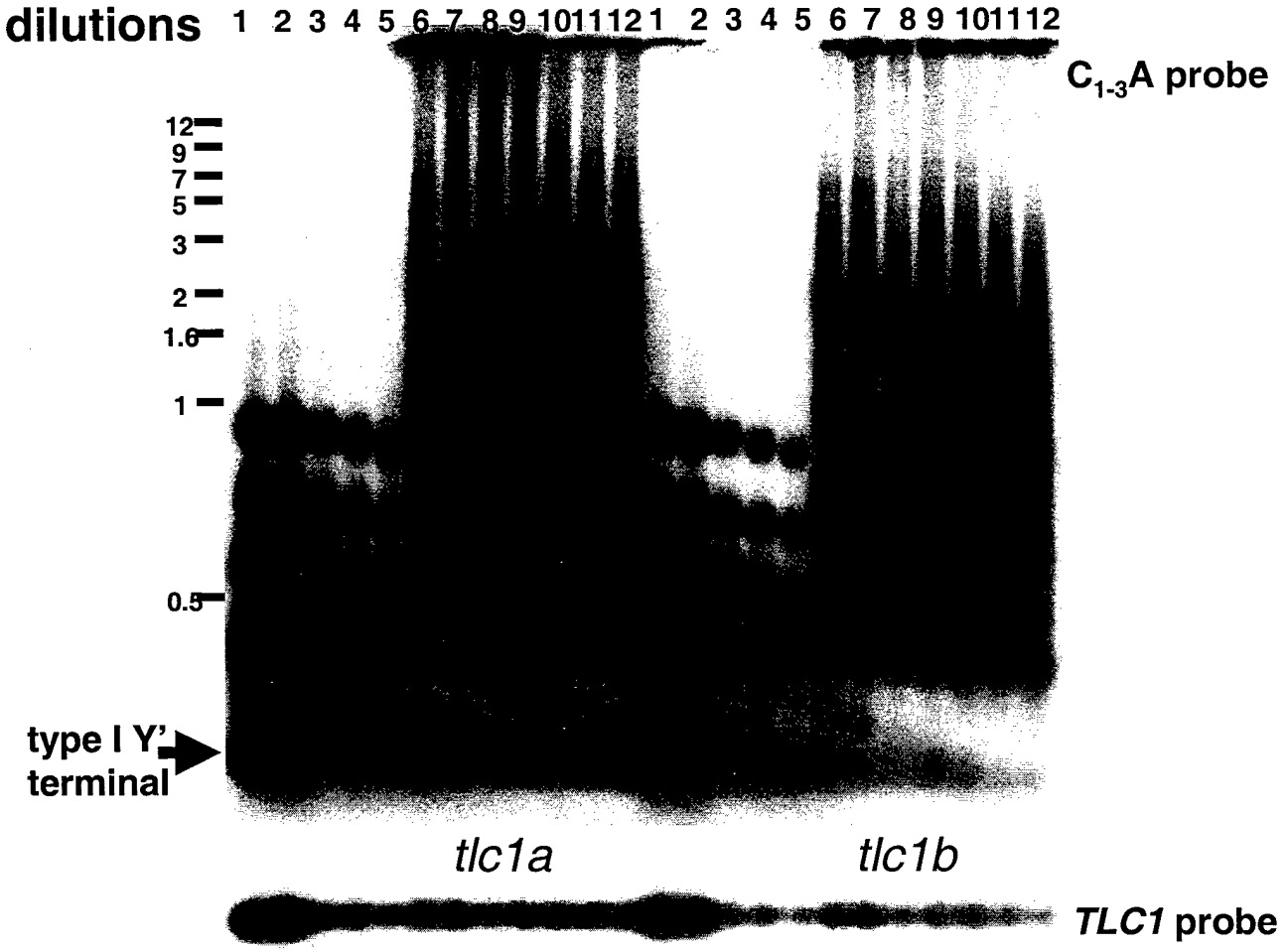
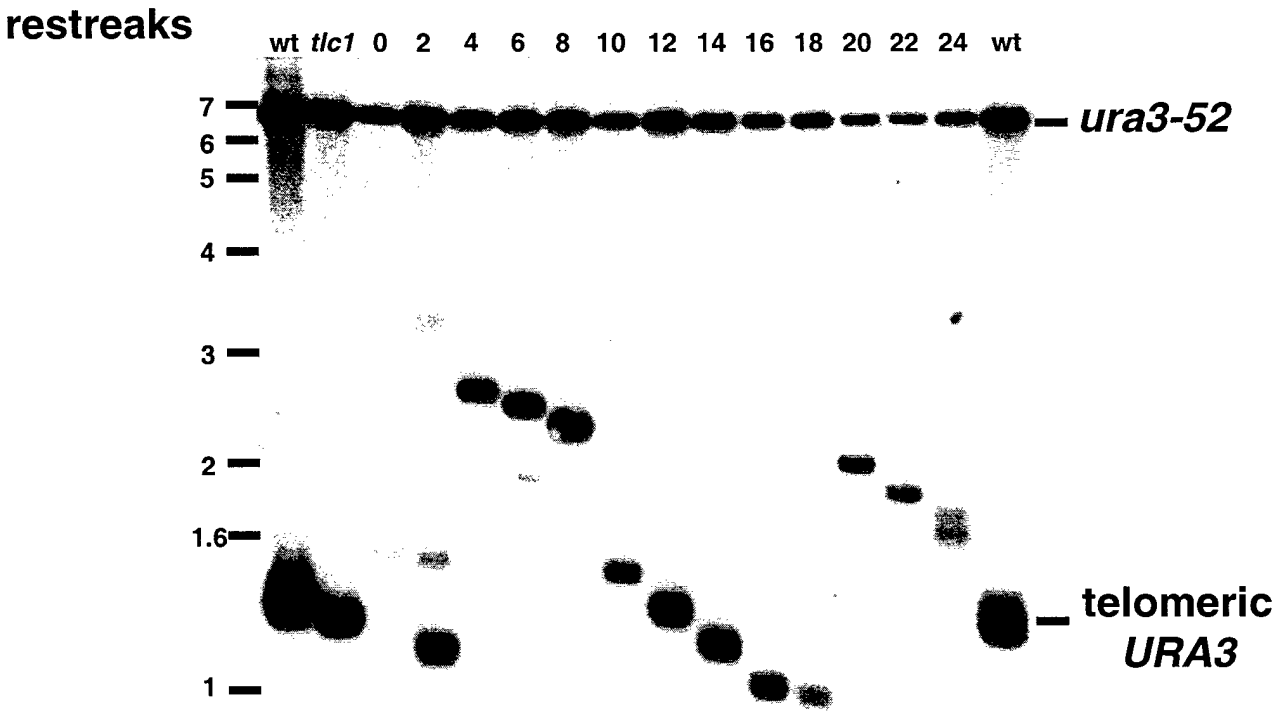


FIGURE 1: One step telomere amplification for type II telomere formation. Two independent freshly dissected *tlc1* spores were used to inoculate YEPD medium. Cultures were diluted 1: 10,000 every 48 hours for 12 times. DNAs were prepared from each culture. The DNA was digested with *Alu*I, *Hae*III, *Hin*fI, and *Msp*I, subjected to electrophoresis in a 1.2% agarose gel, prepared for Southern analysis, and probed with a *S. cerevisiae* $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric probe. The major band from type I telomeric fragments is indicates by the arrow. The positions of molecular weight markers in kilobase pairs are indicated.



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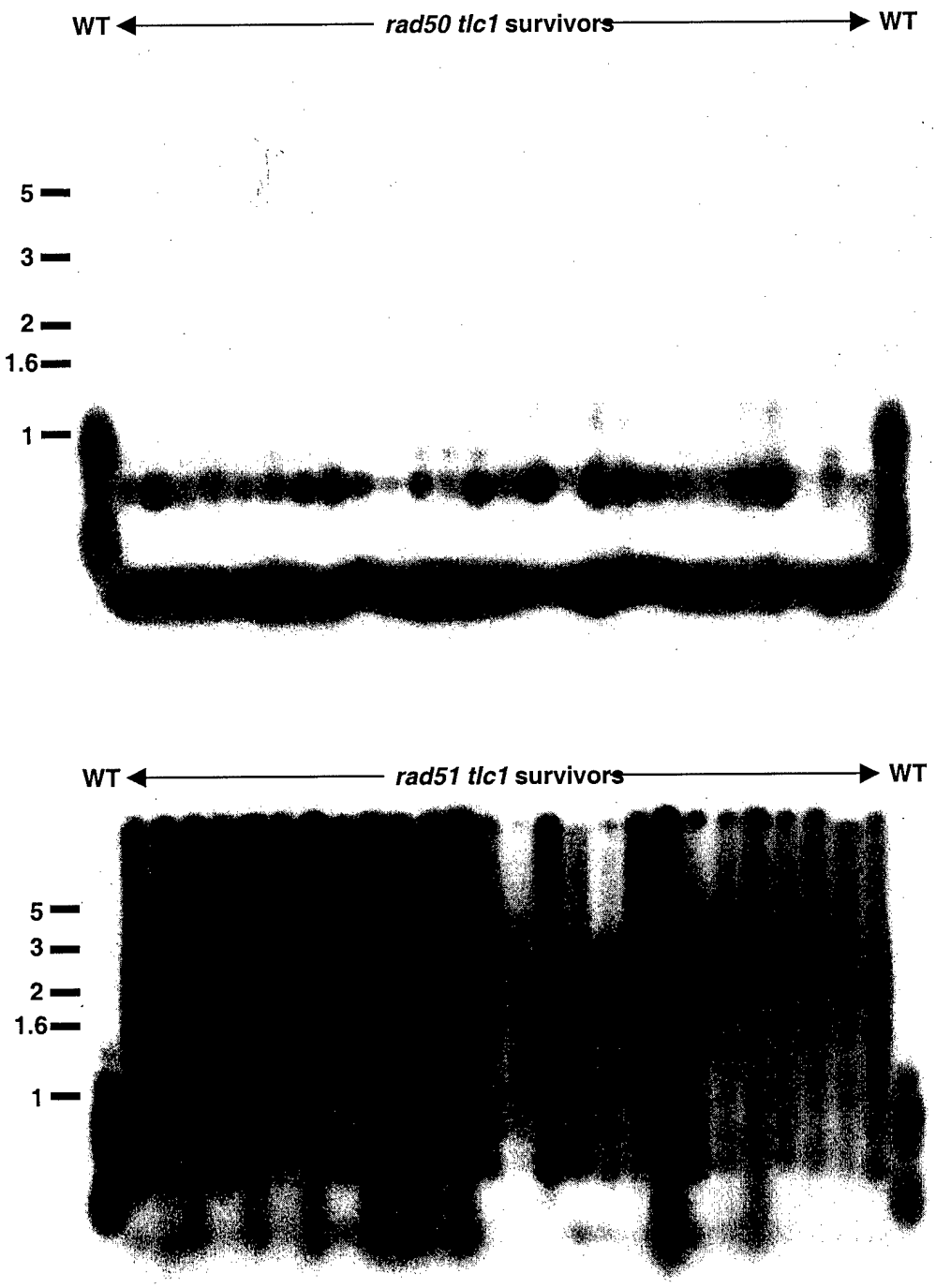

FIGURE 3: All *rad50 tlc1* survivors are type I and *rad51 tlc1* survivors are type II. Survivors were generated by streaking tiny colonies from the re-streak plate. DNA was digested with *Alu*I, *Hae*III, *Hin*fI, and *Msp*I, and subjected to electrophoresis in a 1.2 % agarose gel. Southern blot analysis was performed as in figure 2. 120 survivors from each strain were examined and 26 are shown here.

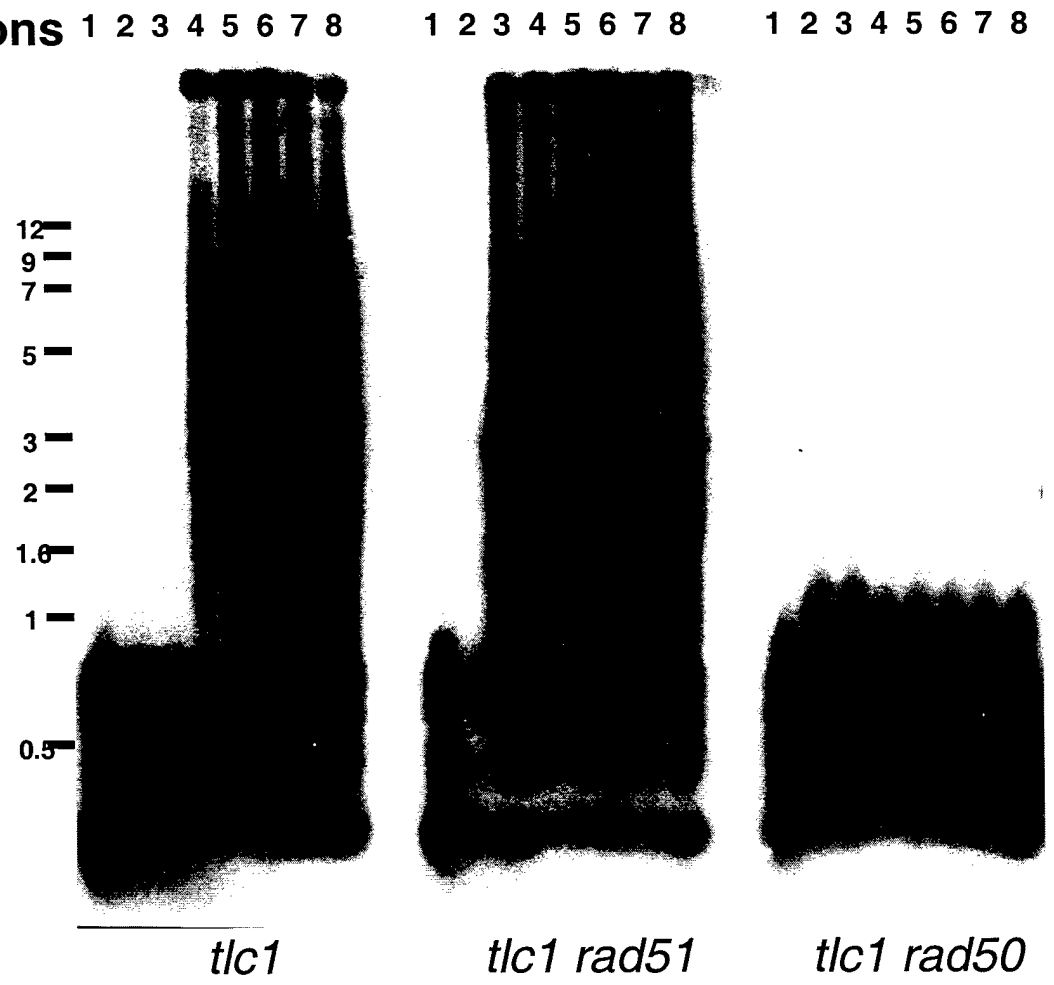

FIGURE 4: Separate roles of *RAD50* and *RAD51* for telomere-telomere recombination. Fresh dissected *tlc1, tlc1 rad51,* and *tlc1 rad50* spores were used to inoculate YEPD medium. Cultures were 1: 10,000 diluted every 72 hours for 8 times. DNAs were prepared from each culture, digested with *Alu*I, *Hae*III, *Hin*fI, and *Msp*I, subjected to electrophoresis in a 1% agarose gel. Southern blot analysis was performed as in figure 2.

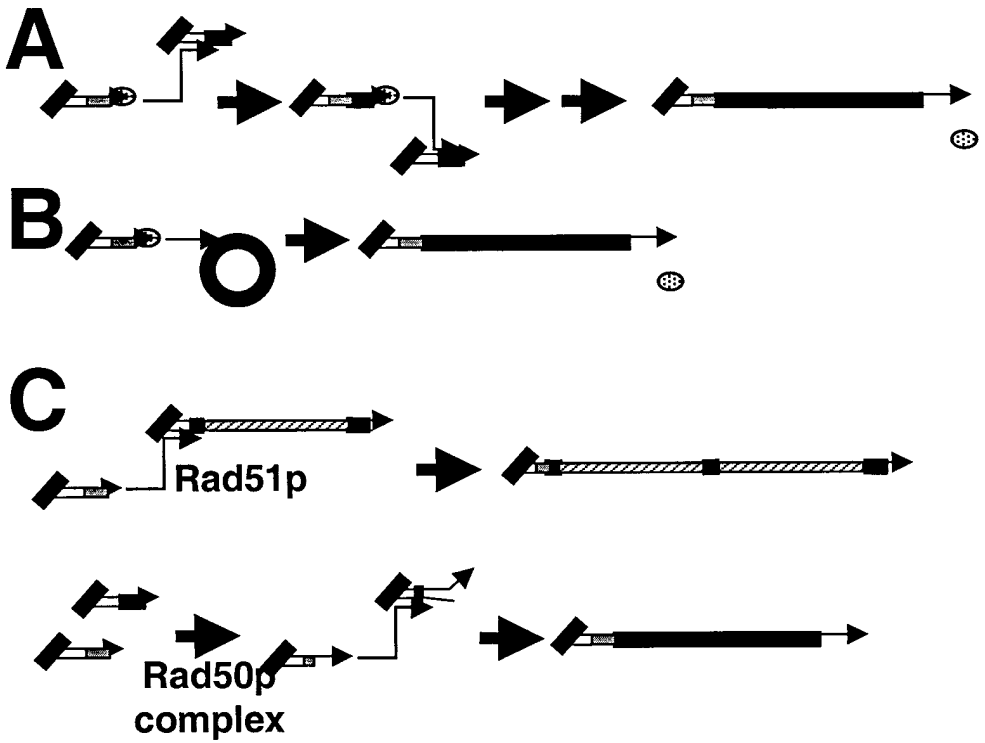

FIGURE 5: (A). Continuous strand transfer using distinct templates. After one round gene conversion, the 3' hydroxyl of the recombination product is re-invaded into the next template before the recombination machinery falls off, so the telomere can continuously recombine using discontinuous templates. **(B). Circular TG_{1-3} ring serving as the template for strand invasion.** A circular telomere ring is pop out from the chromosomal telomere and caught by the 3' hydroxyl primer of the telomere for strand invasion. According to the rolling circle replication model, the telomere can keep being elongated until the polymerase falls off. **(C). Rad50p and Rad51p roles in survivor formation.** See text for details. (The round shaded circle indicates the recombination machinery and the stripped square indicates the Y' element. Donor telomeres for strand invasion are represented as gray boxes and recipient telomeres are represented as black boxes.)

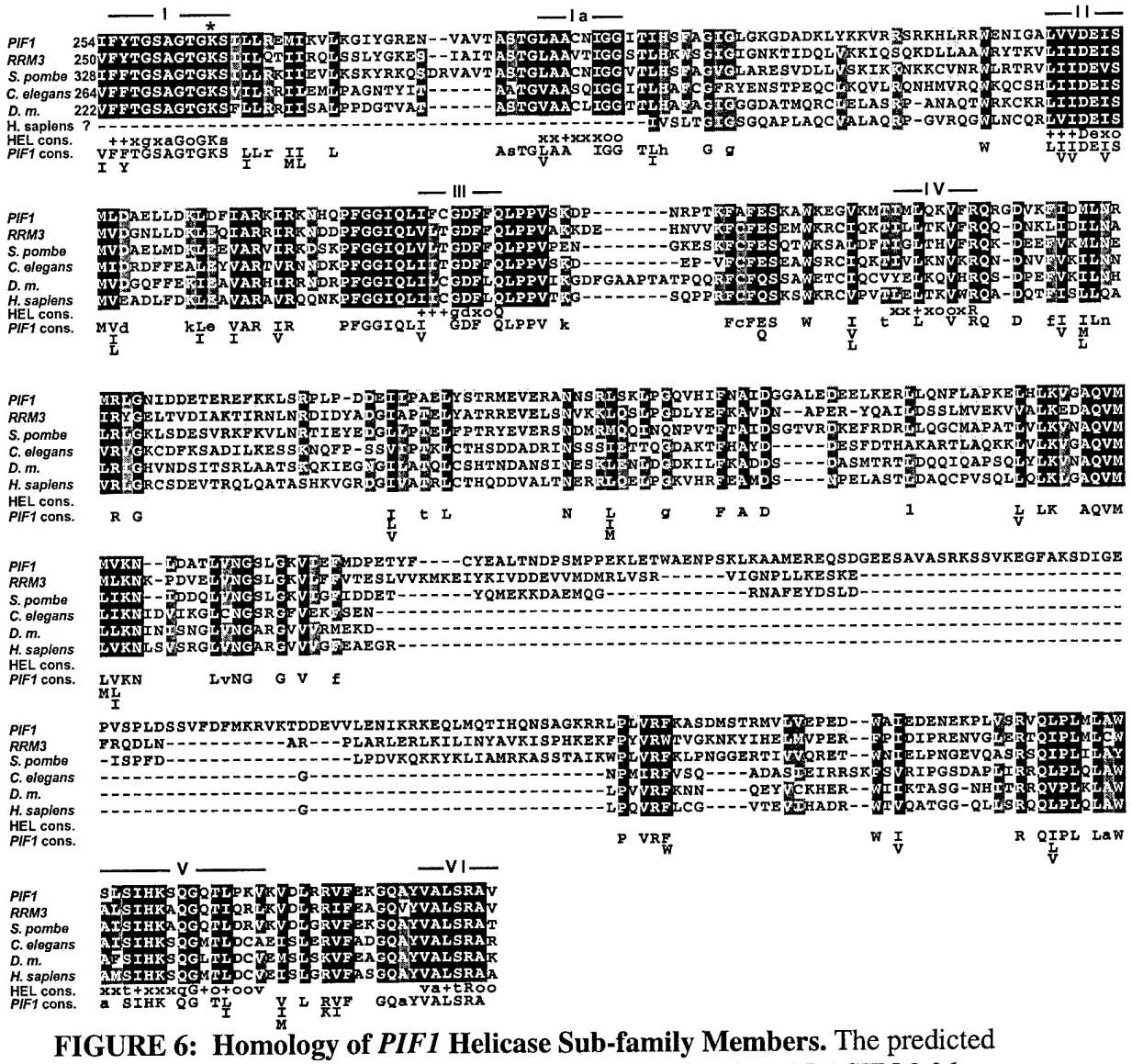

FIGURE 6: Homology of *PIF1* Helicase Sub-family Members. The predicted sequences of the *PIF1*-like proteins were compared using the TBLASTN 2.06 program. The default parameters of the NCBI blast server were used, except that filtering of low complexity regions was not done. The top line for each pair shows the expectation value, a measure of the probability that the match occurred by chance. The number of amino acids of homology shared between the two proteins and percentage identity within the helicase region is shown below. These numbers were obtained by aligning the helicase region using the MacVector 6.0 (Oxford Molecular) implementation of the CLUSTALW program. As determined, an insertion in either of the two proteins counts in the length calculation and reduces the level of identity. The *PIF1*-like proteins, including a fragment of human sequence, were aligned using the MACAW 2.0.5 program. The default parameters were used, including the BLOSUM62 matrix and the segment pair overlap search method. White areas have a mean identity score of 0-37%, grey is 37-67%, and black is 67-100%. The positions of the seven helicase domains are shown above. The *S. pombe* Rph1p is 805 amino acids, the *Saccharomyces* Rrm3p is 723 amino acids, the *Saccharomyces* Pif1p is 857 amino acids, the *C. elegans* sequence is 674 amino acids, the *C. maltosa* partial protein sequence is 365 amino acids, and the human partial protein sequence is 449 amino acids. The peptide region of the 449 amino acids of the known human sequence and the corresponding regions of the other *PIF1*-like proteins were aligned using the MacVector 6.0. Shaded residues are 60% or more identical among all proteins. The positions of helicase domains are shown above the protein sequence.

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Telomere-Telomere Recombination Is an Efficient Bypass Pathway for Telomere Maintenance in *Saccharomyces cerevisiae*

SHU-CHUN TENG AND VIRGINIA A. ZAKIAN*

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

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Many *Saccharomyces* telomeres bear one or more copies of the repetitive Y′ element followed by ~350 bp of telomerase-generated $C_{1-3}A/TG_{1-3}$ repeats. Although most cells lacking a gene required for the telomerase pathway die after 50 to 100 cell divisions, survivors arise spontaneously in such cultures. These survivors have one of two distinct patterns of telomeric DNA (V. Lundblad and E. H. Blackburn, Cell 73:347–360, 1993). The more common of the two patterns, seen in type I survivors, is tandem amplification of Y′ followed by very short tracts of $C_{1-3}A/TG_{1-3}$ DNA. By determining the structure of singly tagged telomeres, chromosomes in type II survivors were shown to end in very long and heterogeneous-length tracts of $C_{1-3}A/TG_{1-3}$ DNA, with some telomeres having 12 kb or more of $C_{1-3}A/TG_{1-3}$ repeats. Maintenance of these long telomeres required the continuous presence of Rad52p. Whereas type I survivors often converted to the type II structure of telomeric DNA, the type II pattern was maintained for at least 250 cell divisions. However, during outgrowth, the structure of type II telomeres was dynamic, displaying gradual shortening as well as other structural changes that could be explained by continuous gene conversion events with other telomeres. Although most type II survivors had a growth rate similar to that of telomerase-proficient cells, their telomeres slowly returned to wild-type lengths when telomerase was reintroduced. The very long and heterogeneous-length telomeres characteristic of type II survivors in *Saccharomyces* are reminiscent of the telomeres in immortal human cell lines and tumors that maintain telomeric DNA in the absence of telomerase.

Telomeres, the protein-DNA structures found at the natural ends of eukaryotic chromosomes, are required to protect chromosomes from degradation and end-to-end fusion and to facilitate their complete replication. In most organisms, telomeric DNA consists of a short, tandemly repeated sequence that has clusters of G residues in the strand that runs 5′ to 3′ toward the chromosome end. For example, *Saccharomyces* chromosomes end in ca. 350 ± 75 bp of $C_{1-3}A/TG_{1-3}$ DNA (see Fig. 1A). In addition, many eukaryotes have middle repetitive DNA elements or telomere-associated (TA) sequences immediately internal to the simple repeats. In *S. cerevisiae*, there are two such sequences, X and Y′. X is a heterogeneous sequence found at virtually all telomeres (25). Y′ is found in one to four tandem copies, immediately internal to the $C_{1-3}A/TG_{1-3}$ repeats, on about two-thirds of yeast telomeres (10, 48). There are two classes of Y′ elements, Y′-short and Y′-long, with the 5.2-kb Y′-short differing from the 6.7-kb Y′-long by a 1.5-kb internal deletion (25). When Y′ is tandemly repeated, a given array consists of all Y′-long or all Y′-short elements (24). In wild-type cells, Y′ sequences can be lost or duplicated by mitotic recombination between sister chromatids or different chromosome ends (24).

In most eukaryotes, including yeast, telomere replication is carried out by a special reverse transcriptase, telomerase, that uses a small C-rich stretch in its RNA component as a template for the extension of the G-rich strand (reviewed in reference 36). The genes encoding the RNA (*TLC1*) (44) and protein catalytic subunit (*EST2*) (12, 22) of the *Saccharomyces* telomerase have been identified. Several additional genes, including *EST1*, which encodes a telomerase RNA-associated protein (20), and *CDC13*, which encodes a protein that binds telomeres in vivo (4), are also required for telomerase replication in vivo (19, 21, 35). When any of the yeast genes that are essential for the telomerase pathway are deleted, the telomere length gradually shortens, chromosome loss increases, and most cells die (see, for example, reference 28).

Telomerase is not the only mechanism that can maintain telomeric DNA. In *Drosophila*, transposition of telomere-specific retrotransposons is the major pathway for telomere maintenance (2). Both telomerase and transposition contribute to telomere maintenance in the green alga *Chlorella* (17). Telomere-telomere recombination is thought to be the sole mechanism for maintaining the repeats at chromosome ends in some insects, such as the mosquito *Anopheles* (40) and the dipteran *Chironomus* (23).

Even in organisms that normally rely on telomerase, telomerase-independent mechanisms of telomere maintenance exist. Although most cells in *S. cerevisiae* (27), *Schizosaccharomyces pombe* (34), and *Kluyveromyces lactis* (29) that lack the gene for a telomerase component die, survivors arise relatively frequently in all three organisms. In both *S. cerevisiae* and *K. lactis*, generation of survivors requires *RAD52*-dependent recombination. In *S. cerevisiae* (discussed in more detail below), the survivors that have been characterized in detail have very short telomeric $C_{1-3}A/TG_{1-3}$ tracts but long tandem arrays of Y′ DNA. In contrast, in *K. lactis*, survivors have long tracts of telomeric repeats (29). *S. pombe* can escape the telomerase requirement in two ways, by amplification of its TA repeats, presumably by recombination, or by loss of both TA and telomeric DNA followed by end-to-end fusions to generate circular chromosomes (33). Since some human cell lines (7) and tumors (8) that lack telomerase have very long telomeres, telomerase bypass pathways exist in mammals as well.

The generation of survivors in the absence of telomerase has been studied most extensively in *est1*Δ strains of *Saccharomyces* (27). In that pioneering study, the authors described two types of telomerase-independent survivors based on the pattern of

* Corresponding author. Mailing address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544. Phone: (609) 258-6770. Fax: (609) 258-1701. E-mail: vzakian@molbio.princeton.edu.

restriction fragments produced after digestion with *Xho*I. Type I survivors had tandem duplication of the subtelomeric Y′ element, whereas type II survivors were suggested to arise by rearrangement and/or tandem duplication of the distal portion of Y′. Similar type I and type II survivors were observed in *tlc1*, *est2*, *est3*, and *est4* strains, but the structure of DNA in these strains has not been characterized in detail (19).

We reinvestigated the structure of telomeric DNA in type II survivors arising in a *tlc1* strain. We found that cDNA-mediated recombination of Y′ elements to chromosome ends occurred but its frequency was too low to support telomere maintenance in the absence of telomerase. Likewise, type II survivors did not arise as a result of chromosome circularization. Rather, type II survivors had very long terminal tracts of $C_{1-3}A/TG_{1-3}$ DNA, with some telomeres being as much as 12 kb longer than telomeres in wild-type cells. This pattern is similar to the exceptionally long telomeres in human tumors (8) or cultured cells (7) that lack telomerase. The maintenance of these elongated telomeres required Rad52p, but reintroduction of telomerase resulted in the slow loss of telomeric DNA until all telomeres returned to wild-type lengths.

MATERIALS AND METHODS

Plasmids, yeast strains, yeast transformations, and genetic manipulation. All the yeast operations were performed by standard methods (39). Yeast strains used in this study were derivatives of YPH501 (*MATa*/*MATα ura3-52*/*ura3-52 lys2-801 amber*/*lys2-801 amber ade2-101 ochre*/*ade2-101 ochre trp1Δ63*/*trp1Δ63 his3Δ200*/*his3Δ200 leu2-Δ1*/*leu2-Δ1*) (43). YPH501 *tlc1::LEU2*/*TLC1* was constructed by transforming *Xho*I-digested pBlue61::*LEU2* (kindly provided by D. Gottschling) (44) into YPH501 and selecting Leu^+ transformants. To complement a *tlc1* strain, a *CEN* plasmid containing *TLC1* was made. The full-length *TLC1* gene plus 1 kb of 5′-flanking and 0.5 kb of 3′-flanking sequences was cloned into pRS317, a vector having *LYS2* as a selectable marker (43). pRS317*TLC1* was transformed into YPH501 *tlc1::LEU2*/*TLC1* and sporulated. YPH *tlc1::LEU2* segregants carrying pRS317*TLC1* were selected by growth on complete medium lacking leucine and lysine.

The *his3AI*-5′, *URA3*, and *his3AI*-3′ were amplified by PCR with, respectively, *his3AI*-5′,5′ (GGACTAGTGCTGCAGCTTTAAATATCG) and *his3AI*-5′,3′ (CCCGCTCGAGATGGTCCTCTAGTACACTC), *URA3*,5′ (CCCGCTCGAG CTTTTCAATTCAATTCATC) and *URA3*,3′ (CTCCCCGCGGGTAATAACT GATATAAT), and *his3AI*-3′,5′ (CTCCCCGCGGTGTCACTACATAAGAAC) and *his3AI*-3′,3′ (TGCTCTAGATGGTCCTCTAGTACTCTC) as primers (underlined segments indicate restriction sites) and pTy*his3AI* (45) as a template. To make pSL300*his3AI-URA3-his3AI*, *Spe*I-*Xho*I-digested *his3AI*-5′, *Xho*I-*Sac*II-digested *URA3*, and *Sac*II-*Xba*I-digested *his3AI*-3′ PCR-amplified fragments were cloned sequentially into the multiple-cloning sites of pSL300 (6). The fragment for tagging the 3′ untranslated region of the Y′ elements with *his3AI-URA3-his3AI* was amplified by PCR with 50-bp Y′ sequences that spanned the stop codon of the Y′ ORF2 at the ends of the primers and pSL300*his3AI-URA3-his3AI* as a template. The resulting Y′-*his3AI-URA3-his3AI*-Y′ PCR-amplified fragment was transformed into the YPH *tlc1::LEU2* strain carrying pRS317*TLC1*. Y′-*his3AI-URA3-his3AI*-tagged strains were selected on medium lacking uracil. Cells that had lost the *URA3* gene by popout recombination were selected on 5-fluoroorotic acid (5-FOA) (3). Tagging of individual telomeres by *his3AI* was confirmed by Southern blot analysis with Y′ and *his3AI* probes as described below.

Formation of survivors. To lose the pRS317*TLC1* plasmid, cells were grown on yeast extract-peptone-dextrose (YEPD) plates overnight and then replica plated to α-aminoadipate plates to identify Lys^- cells (11). Single colonies were restreaked on α-aminoadipate plates. Colonies from α-aminoadipate plates were then streaked on YEPD plates for single-colony purification. This procedure was repeated five times on YEPD plates to allow cellular senescence to occur and survivors to appear. The plates were incubated at 30°C for 3 days. Survivors first appeared after four restreaks on YEPD plates. Alternatively, survivors were obtained by inoculating single colonies from the α-aminoadipate plates into 10 ml of YEPD medium, growing these to stationary phase by incubation at 30°C for 3 days, and then diluting the cultures 1:10,000 into fresh YEPD medium. This procedure was repeated three or four times, and then the cells were plated on YEPD plates to identify survivor colonies. Most survivors obtained by the liquid growth method were type II survivors due to their faster growth compared to type I cells.

To determine if maintenance of survivors required Rad52p, YPH500 (43) was mated to Y0025 (12) (from R. Weinberg), a strain in which the *RAD52* gene was replaced with *HIS3*. One copy of *TCL1* was replaced with *TRP1*, and a centromere plasmid containing *URA3* and *RAD52* was introduced by transformation. This strain was sporulated, and *tlc1:TRP1 rad52:HIS3* spores containing the *RAD52* plasmid were identified. Suppressors were isolated as described above, and then cells that lost the *RAD52* plasmid were identified by their ability to grow on FOA medium.

DNA preparation, enzyme digestion, Southern blot analysis, and gel electrophoresis. Genomic DNA preparation and Southern blot analysis were performed as previously described (30). S1 nuclease and mung bean nuclease treatments were performed as specified by the manufacturer (New England BioLabs). Two-dimensional gel electrophoresis (5) and alkaline denaturing gel electrophoresis (41) were performed as described previously. For the *Bal*31 exonuclease digestion experiment, 70 μg of genomic DNA from wild-type or type II survivors was digested with 3 U of *Bal*31 (New England BioLabs) in a 100-μl final volume. A 14-μl volume of digested DNAs was removed every 10 min, subjected to phenol-chloroform extraction and ethanol precipitation, and digested with *Xho*I. The following probes were used for Southern hybridization: a 270-bp $C_{1-3}A$ fragment, a 1.5-kb *Sph*I-*Sal*I fragment from the 5′ end of Y′, a 4.2-kb *Sal*I-*Xho*I fragment from the middle region of Y′, a 341-bp *Xho*I-*Kpn*I fragment from the 3′ end of Y′, a 586-bp *Nde*I-*Nsi*I fragment of *HIS3*, a 1-kb 5′-*Eco*RI fragment of *PIF1*, and a 350-bp PCR fragment of *TLC1*. Probes were randomly labeled with the RTS-Rad prime system (Life Technologies).

For pulsed-field gel electrophoresis (PFGE), yeast chromosomal DNA blocks were prepared by mixing equal volumes of yeast cells from stationary-phase cultures with 1% low-melting agarose (FMC BioProducts) as described previously (39). PFGE was performed with the contour-clamped homogeneous electric field-dynamically regulated CHEF-DR III system (Bio-Rad). Chromosomes were separated on a 1% agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer at 14°C for 30 h at 6.0 V/cm (200 V) with a 120° included angle and a 60- to 120-s linear switch time ramp.

Inverse PCR, cloning and sequencing. Portions (3 μg) of genomic DNA from wild-type and two independently isolated type II survivors with *his3AI*-tagged telomeres were digested with *Xho*I. The *Xho*I-digested fragments were made blunt ended by using Klenow enzyme in the presence of all four nucleotides. Half of each reaction mixture was subjected to Southern blot analysis to determine the size of the *his3AI*-tagged *Xho*I-digested telomere fragments. The other half of the reaction mixture was separated on a 0.6% gel. DNAs in the correct size range to contain the *his3AI*-tagged *Xho*I-digested telomere fragments were gel purified and ligated at 14°C overnight in a total volume of 200 μl. PCR was carried out with 4 μl of the ligation mix. The PCR conditions were 30 to 40 cycles of 30 s of 94°C denaturation, 1 min of 69°C annealing, and 3 min of 72°C extension. The primers were P1 (5′-TAGCGACCAGCCGGAATGCTTGG-3′) and P2 (5′-AC GATGTTCCCTCCACCAAAGGTG-3′) facing opposite to each other in *his3AI* (see Fig. 6). A further PCR amplification step was performed with nested primers P3 (5′-AGCGCTCGTCATGGAACGCAAAC-3′) and P4 (5′-CGAGAGTA GAGGTAGATGTGAGAG-3′) facing opposite to each other in the Y′ element (see Fig. 6). The PCR-generated products were cloned into the pCRIITOPO vector (Invitrogene) and transformed into *Escherichia coli* STAB2 cells (GIBCO-BRL). Sequencing was performed with cycle-sequencing kits (Epicentre Technologies) with primers P3 and P4.

RESULTS

Identification of survivors in a *tlc1* strain. Although most *est1* cells die, telomerase-independent survivors appear after ~50 to 100 generations (27). This previous study identified two types of survivors in an *est1* strain that are distinguishable by their pattern of telomeric *Xho*I fragments. There is a single site for *Xho*I in Y′ (Fig. 1A). The majority (63%) of *est1* survivors (called type I survivors in this paper) have three major *Xho*I fragments that hybridize to the 3′ Y′ probe (Fig. 1A), which detects the distal portion of Y′. The sizes of these bands are ~1.3, 6.7, and 5.2 kb. The ~1.3-kb fragment is the terminal fragment from Y′ telomeres and consists mainly of Y′ DNA with a very short stretch of $C_{1-3}A/TG_{1-3}$ DNA. The strong hybridization at 6.7 and 5.2 kb is due to tandemly repeated Y′ long and Y′ short elements, respectively (Fig. 1A). In contrast, *Xho*I digestion of DNA from type II survivors yields many differently sized *Xho*I fragments that hybridize to both $C_{1-3}A/TG_{1-3}$ and 3′ Y′ probes but not to probes from other regions of Y′ (27). The pattern of telomeric *Xho*I fragments varies among independent type II survivors. The authors concluded that telomeres of type II survivors sustain substantial Y′ deletions and rearrangements, possibly containing tandem duplications of the distal segment of Y′ (27).

To further understand telomerase-independent mechanisms for telomere maintenance in *S. cerevisiae*, a strain lacking *TLC1*, the gene encoding the RNA component of telomerase

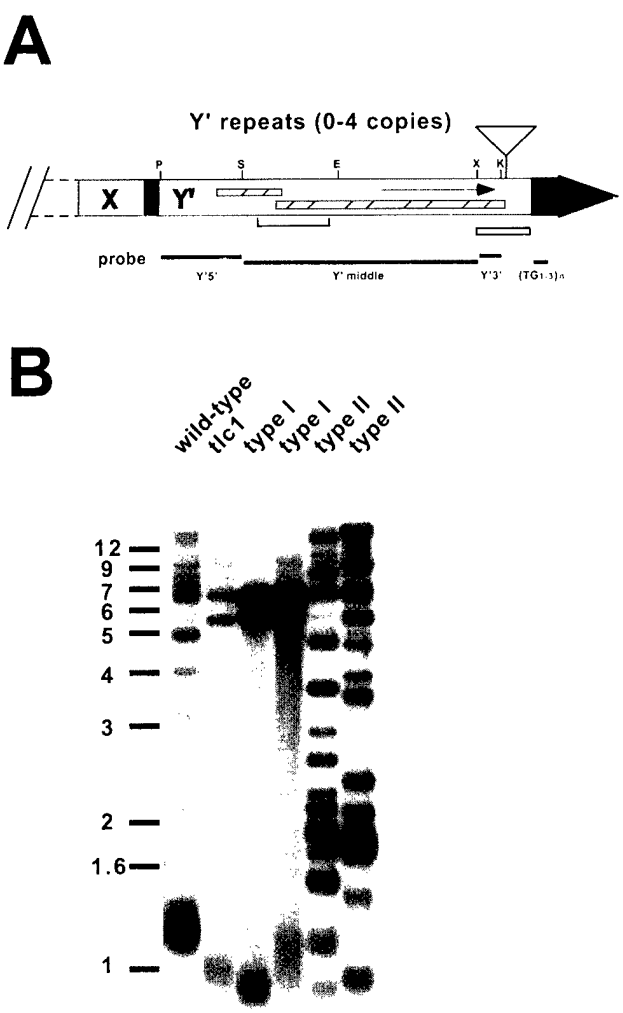

FIG. 1. (A). Telomeric and subtelomeric structure of *S. cerevisiae*. $C_{1-3}A/TG_{1-3}$ DNA is shown in black. The Y′-long element is between a ~50- to 100-bp internal stretch of $C_{1-3}A/TG_{1-3}$ DNA (26, 46) and a 300- to 400-bp terminal $C_{1-3}A/TG_{1-3}$ tract. The two open reading frames in Y′ are shown as striped boxes, and their direction of transcription is indicated by the arrow. The position of the 0.9-kb *his3A1* insert is shown as a triangle. The deleted region in Y′-short is indicated by brackets (25). The 3′ end of Y′ is shown as an open rectangle. The solid lines indicate the restriction fragments used as probes. Restriction sites used for Southern blot analysis are as follows: P, *Sph*I; S, *Sal*I; E, *Eco*RI; X, *Xho*I; K, *Kpn*I. (B). Telomeres of *tlc1* survivors show two distinct patterns. Genomic DNA from the wild type, senescing *tlc1* cells, two independent type I *tlc1* survivors, and two independent type II *tlc1* survivors was digested with *Xho*I, fractionated through 1% agarose, and analyzed by Southern blotting with a $C_{1-3}A/TG_{1-3}$ probe. Size markers are in kilobases.

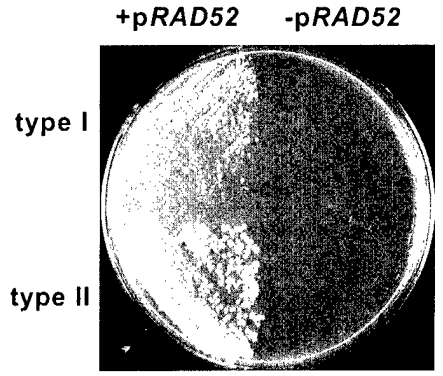

FIG. 2. The maintenance of type I and type II survivors requires Rad52p. Eight independent type I and type II survivors were isolated in a *rad52 tlc1* strain carrying a plasmid bearing the *RAD52* gene. After survivors were characterized, the *RAD52* plasmid was lost and cells were streaked onto rich medium. Shown here are cells from one type I and one type II survivor with (left) or without (right) the *RAD52* plasmid. The plate is the first restreak after plasmid loss. Some type II survivors grew on the first restreak but not on subsequent streaks. This figure also demonstrates that type II survivors grew faster than type I survivors (compare streaks of +p*RAD52* for type I and type II).

was created. This strain contained a *LYS2* CEN plasmid harboring the wild-type *TLC1* gene to complement the chromosomal *tlc1* deletion. Cells that lost the *TLC1* plasmid were identified and then restreaked multiple times to obtain survivors. Although most *tlc1* cells died, faster-growing survivor cells appeared after ca. 100 to 125 generations (data not shown). Genomic DNA from 24 independent survivors, as well as from wild-type and early-passage *tlc1* strains, was isolated, digested with *Xho*I, and analyzed by Southern blotting with a $C_{1-3}A/TG_{1-3}$ probe. Most (21 of 24) survivors were type I (two examples are shown in Fig. 1B, lanes type I), and three were type II (lanes type II). Additional type II survivors were obtained in independent experiments.

The growth characteristics of nine type I and nine type II survivors were analyzed by restreaking each survivor 10 times on YEPD plates. The growth rate of most type II survivors and wild-type colonies was similar, whereas type I survivors grew more slowly and their growth rate fluctuated in different restreaks (see, for example, Fig. 2), with senescing cells reappearing at different times during outgrowth. Type I and type II survivors from an *est1* strain had similar growth properties (27). We conclude that the survivors obtained in a *tlc1* strain were indistinguishable from those obtained in an *est1* strain.

Survivors require the continuous presence of Rad52p. Survivors are not obtained in either *rad52 est1* strain (27) or *rad52 tlc1* strains (18). To determine if Rad52p is required to maintain survivors in cells lacking telomerase, we isolated eight independent type I and eight independent type II survivors in a *rad52Δ tlc1* strain carrying a *RAD52 URA3* plasmid. After survivors were generated, cells that lost the *RAD52* plasmid were identified by their ability to grow on plates containing 5-FOA. Type I survivors grew very poorly or not at all, even on the first restreak after loss of the *RAD52* plasmid (Fig. 2). Although type II *tlc1 rad52* survivors divided more times than type I *tlc1 rad52* survivors did, they formed heterogeneously sized colonies similar to those seen in senescent *tlc1* cells and stopped growing altogether after one to three restreaks (Fig. 2). Thus, *RAD52* function is needed continuously to maintain both type I and type II survivors.

Telomeres of *tlc1* survivors are not maintained through cDNA-mediated recombination. *Drosophila* telomeres consist of retrotransposons (1, 17). Y′ elements share several structural features with retrotransposons, such as having two overlapping open reading frames that are in different frames but oriented in the same direction (14, 25). Moreover, many integrated copies of retrotransposons have truncated 5′ ends due to premature termination of reverse transcription (14), a situation that could explain the proposed tandem duplication of just the 3′ end of Y′ (27). In yeast, a chromosome without a telomere is seen as a double-strand break (42) and retrotransposons are able to repair chromosomal breaks (31, 45). The *RAD52* dependence for generating survivors would be explained if Y′ cDNAs were added to chromosome ends by

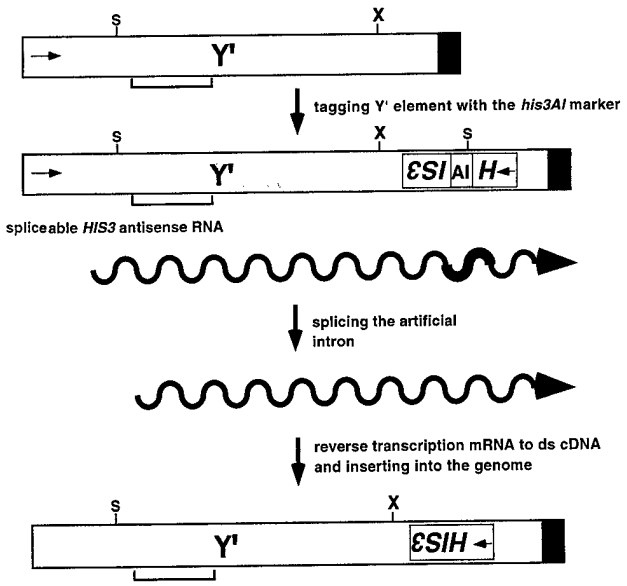

FIG. 3. The *his3AI* gene can be used to detect reverse transcriptase-mediated addition of Y′ to chromosome ends. The *his3AI* gene was inserted within the 3′ end of Y′ (Fig. 1A). The *his3AI* gene is transcribed from the Y′ promoter to generate an antisense *HIS3* transcript having the *AI* intron in a spliceable orientation. If this transcript is spliced and reverse transcribed, it will generate a cDNA containing an uninterrupted *HIS3* gene flanked on either side by Y′ sequences. This Y′ DNA can direct the recombination of the cDNA to a chromosome end. *HIS3* gene expression is detected by growth on plates lacking histidine. The deleted region in Y′-short is denoted by brackets. Restriction sites used for Southern blot analysis are indicated: S, *Sal*I; X, *Xho*I. ds, double-stranded.

homologous recombination. These considerations led us to test if type II survivors are generated by RNA-mediated transposition of Y′ DNA.

To test this possibility, we first tagged the 3′ end of Y′ elements with the *his3AI* marker (13) (Fig. 3). The *his3AI* gene is designed to detect reverse transcription-mediated events in yeast. In *his3AI*, the *HIS3* ORF is interrupted by a 104-bp artificial intron (AI) that is oriented opposite to the direction of *HIS3* transcription. Transcription of the *his3AI* gene results in a nonspliceable RNA. The *his3AI* gene was inserted within Y′ such that transcription from the Y′ promoter generates a transcript containing antisense *HIS3* sequences interrupted by the AI intron in a spliceable orientation. Reverse transcription of the spliced RNA, followed by either recombination or transposition of the *HIS3* cDNA, will generate His^+ colonies.

Both wild-type and *tlc1* strains that contained *TLC1* on a plasmid were transformed with a construct having the *his3AI* marker inserted into the middle of the 0.9-kb 3′ end of Y′ DNA at the downstream boundary of ORF2 (Fig. 3). Transformants were screened by Southern blotting to obtain strains that had a single *his3AI*-tagged telomere. Since different telomeres have zero to four copies of Y′, the *his3AI* marker could insert within either an internal or a terminal Y′ element. We recovered eight wild-type strains, of which three had the *his3AI* gene inserted within an internal Y′ and five had it inserted within a terminal Y′. We recovered 14 *tlc1* strains containing the *TLC1* plasmid, comprising 4 tagged at an internal Y′ element and 10 tagged at a terminal Y′ element.

We selected for loss of the *TLC1* plasmid in *tlc1* cells containing a *his3AI*-tagged telomere by using the liquid assay described in Materials and Methods. The liquid culture scheme favored the isolation of type II survivors because of their growth advantage compared to type I survivors. Indeed, Southern blot analysis revealed that most survivors (24 of 24 examined) had the *Xho*I restriction pattern diagnostic for type II survivors (data not shown). The cultures were then plated onto YEPD plates to determine the total cell number and onto complete plates lacking histidine to determine the fraction of His^+ cells. If type II survivors are generated via a cDNA intermediate, the majority of them should have a His^+ phenotype. Three *tlc1* His^+ colonies were recovered from a total of 4.2×10^9 postsenescent *tlc1* cells. No His^+ colonies were identified in 6.6×10^9 wild-type cells. The three His^+ colonies from the *tlc1* survivors contained a 0.8-kb *Xho*I-*Nde*I fragment that hybridized to the *HIS3* probe but not to a probe for *AI*, as expected if the His^+ phenotype resulted from cDNA-mediated movement of *HIS3* to a chromosome end. In addition, these strains contained one or more copies of a 0.9-kb *Xho*I-*Nde*I fragment that hybridized to both the *HIS3* and *AI* probes, as expected for the original *his3AI* locus. We conclude that cDNA-mediated movement of a *his3AI*-tagged Y′ occurred but at too low a rate to explain the formation of type II survivors. A similarly small number of His^+ cells was found among type I survivors.

Terminal but not internal Y′ elements are altered during generation of type II survivors. We used strains having a single *his3AI*-tagged telomere to determine the structure of telomeric DNA in type II survivors. Twelve independent *tlc1* strains (strains 1 to 12), each with a single *his3AI*-tagged Y′ element, were generated. Of the 12 strains, 3 (strains 1, 2, and 6) had *his3AI* at an internal Y′. Strain 1 was marked at an internal Y′-short element, and strains 2 and 6 were marked at an internal Y′-long element. The nine other *tlc1* strains had *his3AI* inserted within a terminal Y′ element. Each of the 12 strains was diluted into liquid YEPD medium to generate survivors. Two different survivors (a and b) from each of the 12 strains were examined in detail.

Genomic DNA was prepared from each of the 24 survivors, digested with *Xho*I, and examined by Southern blotting with probes for telomeric $C_{1-3}A/TG_{1-3}$ DNA (Fig. 4A), the 3′ end of Y′ (Fig. 4B), the 5′ and middle parts of Y′ (data not shown), and *his3AI* (Fig. 4C). The telomeric and 3′ Y′ probes detected multiple irregularly sized bands that ranged in size from ~1.5 to 10 kb in each of the 24 strains. Most *Xho*I fragments hybridized to both the telomeric and 3′ Y′ probe (Fig. 4A and B) but not to the 5′ or middle regions of Y′ (data not shown). Like type I survivors, some (but not all [see also Fig. 1]) of the type II survivors had elevated levels of tandem Y′-long and Y′-short elements compared to the starting wild-type strain (Fig. 4B). The hybridization pattern reported here is identical to that described previously for type II survivors (27). Thus, 24 of 24 recovered survivors were type II survivors. Given the growth advantage of type II over type I survivors and the method used to generate survivors, the predominance of type II survivors was expected.

To determine the fate of individual telomeres during survivor formation, the same blot was hybridized with a *his3AI* probe (Fig. 4C). The sizes of the *his3AI*-hybridizing sequences in survivors obtained from *tlc1* strains with *his3AI* inserted into an internal Y′ (strains 1, 2, and 6) were unchanged compared to the starting *tlc1* strain. These bands had the size expected for insertion into Y′-short (strain 1, a and b) or the size expected for insertion into Y′-long (strains 2 and 6, a and b). These data suggest that during formation of type II survivors, internal Y′ elements were not subjected to major rearrangements.

The pattern of *his3AI* hybridization in the 18 survivors obtained from *tlc1* strains that had *his3AI* inserted at a terminal Y′ was diverse. Of the 18 survivors, 12 still had a single *his3AI*-

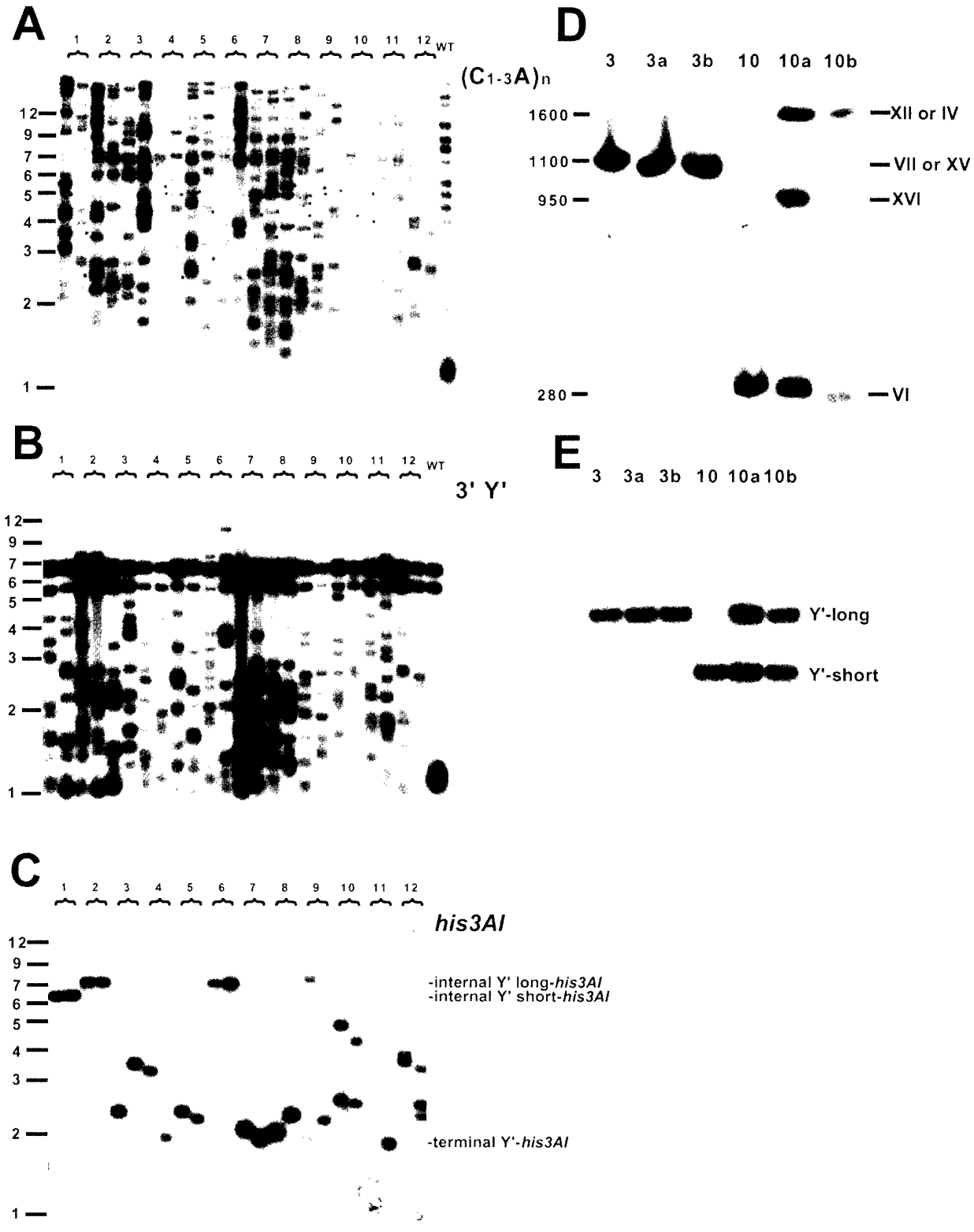

FIG. 4. Southern blot analysis of telomeric DNA in independent type II survivors. (A to C) Two *tlc1* survivors (named a and b) from each of 12 independent *tlc1* strains (strains 1 to 12) with a single *his3AI*-tagged Y′ element were collected. Genomic DNA from each was digested with *Xho*I, fractionated through a 1% agarose gel, and analyzed by Southern blotting. The filter was hybridized sequentially with a $C_{1-3}A/TG_{1-3}$ (A), 3′ Y′ (B), or *his3AI* (C) probe. Most fragments smaller than 5.2 kb that hybridized to the $C_{1-3}A/TG_{1-3}$ probe (A) also hybridized to the 3′ Y′ probe. Fragments in panel A that did not hybridize to the Y′ probe are marked with asterisks. Size markers are in kilobases. (D) Interchromosomal recombination in telomerase-minus *S. cerevisiae*. Chromosomes from two sets of type II survivors (strains 3 a, 3 b, 10 a, and 10 b) and their parent strains (strains 3 and 10) were separated by PFGE. Size markers (in kilobases) are shown on the left. Chromosome numbers are shown on the right (9). (E). Inter-Y′ recombination in telomerase-minus *S. cerevisiae*. Genomic DNAs from the same sets of samples in panel D were digested with *Sal*I. After transfer to a nylon filter, the filters in panels D and E were hybridized with the *his3AI* probe.

tagged telomere, 1 had no tagged telomere (strain 11 a), and 5 had two or more *his3AI* tags (strains 9 a, 10 a and b, and 12 a and b). In no case did the two survivors obtained from the same starting *tlc1* strain have the same structure. The only consistent feature was that the tagged Y′ in the survivor was almost always larger than the tagged Y′ fragment in the parental *tlc1* strain (the position of the parent band is marked terminal Y′-*his3AI* in Fig. 4C). These data indicate that the structure of terminal Y′ elements is usually altered during the formation of a type II survivor.

To obtain a better understanding of the kinds of changes that can occur during formation of type II survivors, we used PFGE to determine the chromosomal location of the *his3AI* tag in four independent type II survivors as well as in their parent strains (Fig. 4D). In strain 3, there was a single copy of *his3AI* inserted within a terminal Y′ element on either chromosome VII or XV (these chromosomes comigrate in PFGE [9]). In this case, the telomere lengthening that accompanied the transition to a type II survivor did not involve movement of *his3AI* to a different chromosome, since the positions of *his3AI* in the two survivors were the same as in the starting strain (Fig. 4D). Strain 10 also had a single copy of *his3AI* within a terminal Y′ element, this time on chromosome VI. Both strain 10 survivors retained *his3AI* on chromosome VI but in addition had *his3AI* on other chromosomes (two new copies of *his3AI* in survivor 10 a, on chromosomes VII and IV or XII; one new copy in survivor 10 b, on chromosome IV or XII). Digestion with *Sal*I can be used to determine if *his3AI* was inserted into Y′-short or Y′-long (Fig. 1A). In strain 3 and its two survivors, *his3AI* was embedded in Y′-long (Fig. 4E). However, during generation of survivors 10 a and 10 b, the *his3AI* tag moved not only to a new chromosome but also from a Y′-short to a Y′-long element (Fig. 4E). These results indicate that both intra- and interchromosomal events occur during generation of type II survivors.

The *his3AI* sequences are still near the physical end of the chromosome in type II survivors. Since the *his3AI* sequences were invariably on larger *Xho*I fragments in type II survivors than in the starting strain (Fig. 4C), it is possible that they were no longer at the ends of linear chromosomes. For example, chromosome ends without a telomere could fuse to form a circular chromosome, as seen in telomerase-minus *S. pombe* (33). If the *his3AI* marker were at the physical end of a chromosome in type II survivors, it would be sensitive to digestion by the exonuclease *Bal* 31. To address this possibility, genomic DNA was prepared from a wild-type strain with *his3AI* inserted within a terminal Y′ (Fig. 5A, left) and from four independent type II survivors (analysis of survivor 3 b is shown in Fig. 5A, right). DNA was digested with *Bal* 31 with samples removed at 10-min intervals. The DNA was then digested with *Xho*I, subjected to electrophoresis, and analyzed by hybridization to a *his3AI* probe. Since the *his3AI* hybridizing sequences shortened at the same rate in both wild-type and type II survivor 3 b DNA, *his3AI* sequences were near a free end in the 3 b survivor. However, it took longer to degrade the *his3AI* sequences in the 3 b survivor DNA than in its parent strain, suggesting that in the type II survivor, the *his3AI* sequences were further from the physical end of the chromosome, a result consistent with the larger size of this fragment. Hybridization of the same blot with the internal *PIF1* probe demonstrated that nontelomeric sequences were not *Bal* 31 sensitive (Fig. 5A). Similar results were obtained with three other type II survivors (data not shown). These data argue that the alterations in *his3AI*-bearing restriction fragments that accompanied the generation of type II survivors do not alter the telomere-proximal location of the tag.

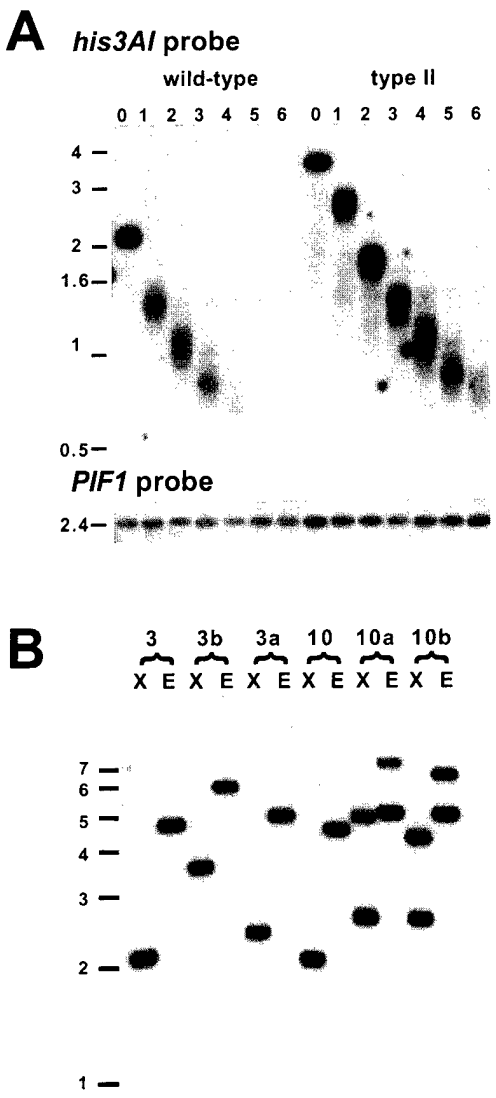

FIG. 5. The *his3AI* gene remains near a free chromosome end and embedded within Y′ DNA in type II survivors. (A) Genomic DNA from the wild-type strain and a type II survivor (survivor 3 b in Fig. 4C) was digested with *Bal* 31 exonuclease for increasing lengths of time. Samples were removed at 10-min intervals, subjected to phenol-chloroform extraction and ethanol precipitation, and digested with *Xho*I. Digested DNAs were fractionated in a 0.7% agarose gel and analyzed by Southern hybridization with, sequentially, a *his3AI* probe (top) and an internal probe (the *PIF1* gene) (bottom). (B) Genomic DNA from two sets of type II survivors (strains 3 b, 3 a, 10 a, and 10 b) and their parent strains (strains 3 and 10) were digested with *Xho*I (X) and *Eco*RI (E) separately. Digested DNA was fractionated in a 0.7% agarose gel and analyzed by Southern hybridization with a *his3AI* probe. Size markers are in kilobases.

The *his3AI* sequences are still embedded in Y′ DNA in type II survivors and have a local structure very similar to that of the starting strain. The *Bal* 31 data suggest that the *his3AI* sequences in a type II survivor were on a terminal *Xho*I fragment but were further from the chromosome end than in the parental *tlc1* strain. Restriction enzyme analysis was used to demonstrate that the DNA surrounding the *his3A* gene in different type II survivors had the characteristics expected for Y′ DNA. Genomic DNA from each of four survivors (strains 3 a, 3 b, 10 a, and 10 b in Fig. 4C) as well as DNA from their parent strains (strains 3 and 10) was digested with five different restriction enzymes, *Sal*I, *Hin*dIII, *Eco*RI, *Bgl*II, and *Kpn*I, all

of which have recognition sites within Y′ and/or *his3AI* DNA (see Fig. 1A for the locations of the sites). Digested DNA was analyzed by Southern blotting with a *his3AI* probe. The data obtained with *Eco*RI are shown in Fig. 5B. The *Xho*I fragments that hybridized to the *his3AI* probe were larger in each type II survivor than the 2.1-kb *Xho*I fragment produced in the parent *tlc1* strains (Fig. 5B). For example, in survivor 3 b, a 3.5-kb *Xho*I fragment hybridized to *his3AI*, whereas in survivor 3 a, a 2.4-kb *Xho*I fragment hybridized to *his3AI* (Fig. 5B). There is an *Eco*RI site in the Y′ element 2.6 kb internal to the *Xho*I site (Fig. 1A). If *his3AI* were still embedded in the same place in Y′ in survivor strains, *his3AI* should hybridize to a 6.1-kb *Eco*RI fragment in survivor 3 b (3.5 + 2.6 kb) and a 5-kb *Eco*RI fragment in survivor 3 a (2.4 + 2.6 kb), exactly the pattern seen (Fig. 5B). Likewise, in survivors 10 a and b, the *Eco*RI fragment(s) that hybridized to *his3AI* were 2.6 kb larger than the *his3AI*-hybridizing *Xho*I fragments. Since this result was obtained with five of five restriction enzymes, the *his3AI* fragments in type II survivors behaved as if they were embedded within Y′ DNA. Thus, we found no evidence for rearrangement or deletion of Y′ elements on the tagged telomeres of type II survivors.

One explanation for the structure of telomeres in type II survivors is that their sequence is similar to that of the starting strain but their structure is altered in a manner that reduces their mobility in agarose gels. For example, type II survivors might have telomeres with very long single-stranded TG_{1-3} tails, as seen in *cdc13-1* cells at semipermissive temperatures (15), and these single-stranded tails might form secondary structures that reduced fragment mobility. However, the mobility of *his3AI*-tagged telomeres did not change after treatment with the single-strand-specific S1 or mung bean nucleases (data not shown). Moreover, analysis by both two-dimensional (5) and alkaline denaturing (41) gel electrophoresis revealed no difference between telomeres of type II survivors and wild-type cells (data not shown). Thus, there was no evidence for any change in the structure of Y′ (Fig. 5B) or in the physical structure of the chromosome end. We conclude that the slower migration of the terminal *Xho*I fragments from *his3AI*-tagged telomeres in type II survivors (Fig. 4C) is probably due to the addition of DNA distal to the tagged Y′ element.

$C_{1-3}A/TG_{1-3}$ DNA is distal to Y′ on *his3AI*-tagged telomeres in type II survivors. An inverse PCR strategy (37) (Fig. 6) was used to obtain the sequence of the DNA to either side of *his3AI* in two type II survivors, 3 a and b, as well as in the parental *tlc1* strain (Fig. 6). Genomic DNA from each of the three strains was digested with *Xho*I, and the restriction fragments were rendered blunt ended by treatment with the Klenow fragment of DNA polymerase I. The *his3AI Xho*I fragments from the parent (2.1 kb), survivor 3 a (2.4 kb), and survivor 3 b (3.5 kb) strains were gel purified and treated with ligase. Because the DNA was dilute, ligation generated intramolecular circles. The circularized *Xho*I fragments were subjected to PCR amplification with primers P1 and P2, which are 395 bp apart and facing in opposite directions within the *his3AI* gene (Fig. 6). For each strain, a PCR product of the expected size was obtained but only in ligase-treated DNA (data not shown). Since there were other ligase-independent PCR products in the first PCR amplification, we did an additional PCR amplification on the products of the first reaction by using nested primers P3 and P4. P3 annealed at the very 3′ end of Y′, just upstream of the telomeric $C_{1-3}A/TG_{1-3}$ telomeric tract. P4 annealed just downstream of the *Xho*I site in Y′ and was oriented toward the *Xho*I site (Fig. 6). Again, PCR fragments of the appropriate size were obtained. These PCR products hybridized to a $C_{1-3}A/TG_{1-3}$ probe (data not shown).

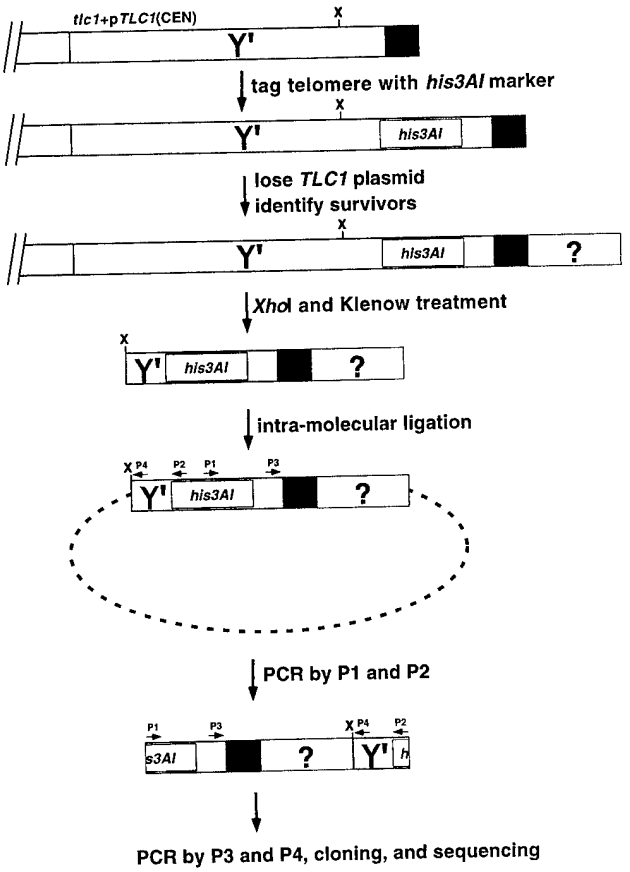

FIG. 6. Inverse PCR strategy to determine the sequence of the DNA flanking the *his3AI* gene in type II survivors. Wild-type and *tlc1* strains with *TLC1* on a plasmid and containing a single *his3AI* tag were identified and then allowed to lose the *TLC1* plasmid. The black box represents the telomeric $C_{1-3}A/TG_{1-3}$ tract present before survivors were formed. The DNA added to the *his3AI* gene during the formation of type II survivors is indicated by the question mark. Genomic DNA from the wild type and type II survivors was digested with *Xho*I and made blunt ended by treatment with Klenow enzyme in the presence of all four nucleotides. The *his3AI*-tagged *Xho*I-digested telomere fragments were gel purified and intramolecularly ligated. Ligated telomeric fragments were subjected to PCR amplification with primers P1 and P2. The P1-P2 PCR products were then subjected to nested PCR amplification with primers P3 and P4. Nested PCR products were further analyzed by cloning and sequencing.

Because the PCR products were from a population of DNA molecules, the exact sequence of telomeric DNA varied from molecule to molecule. Although we could not obtain a precise telomeric sequence from PCR-amplified DNA, when the pool of molecules was sequenced with the P3 primer, the products of all three strains consisted of only T and G residues. When the P4 primer was used, 45 bp of Y′ sequence was followed by DNA consisting of only A and C residues (data not shown).

To determine the precise sequence of an individual telomere, we cloned the products from the second PCR from survivor 3 a and from its parent *tlc1* strain prior to its losing the *TLC1* plasmid. Whereas telomeres from the parent strain were easily recovered, type II survivor telomeres were clonable only in STAB2 *E. coli* (GIBCO-BRL), a strain used to stabilize long tracts of repetitive DNA. We obtained an insert of 0.6 kb, the appropriate size for the telomeric *his3AI* fragment from survivor 3 a. Using the P3 and P4 primers, we sequenced ~100 to 200 bp from each end of the insert and found that it consisted solely of $C_{2-3}A(CA)_{1-6}/(TG)_{1-6}TG_{2-3}$ DNA. Although we did

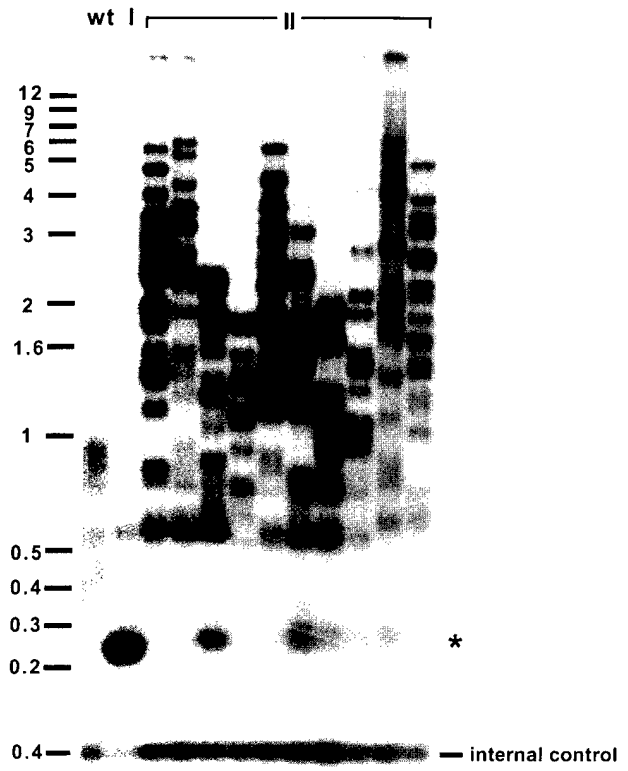

FIG. 7. Newly elongated telomeric fragments in type II survivors are $C_{1-3}A/TG_{1-3}$ tracts. Genomic DNAs from a wild-type strain, a type I survivor, and 10 type II survivors were digested with a combination of *Alu*I, *Hae*III, *Hin*fI, and *Msp*I, which cut at the AGCT, GGCC, GANTC, and CCGG sequences, respectively; fractionated through 1.3% agarose; and transferred to a nylon filter. The filter was hybridized sequentially to a $C_{1-3}A/TG_{1-3}$ probe (top) and a probe for a nontelomeric fragment (taken from a region of *TLC1* that was not deleted; serves as a control for DNA loading). Size markers (kilobases) are shown at the left. An asterisk marks the position of critically short telomeres.

not obtain a precise sequence for the rest of the tract, it had the appropriate pattern to be $C_{1-3}A/TG_{1-3}$ DNA. Thus, a combination of restriction digestion (Fig. 5B) and sequence analysis indicates that the structure of the *his3AI*-tagged telomere in survivor 3 a was unchanged from the same telomere in the parent strain except that its telomeric $C_{1-3}A/TG_{1-3}$ tract was much longer.

Type II survivors have very long tracts consisting only of $C_{1-3}A/TG_{1-3}$ DNA. The cloning and sequencing results confirmed that the *his3AI* marker in survivor strain 3 a was still embedded at the same site within Y′DNA as in the starting strain and that it had ~600 bp of $C_{1-3}A/TG_{1-3}$ DNA distal to Y′. This result suggests that the structure of telomeres in type II survivors is similar to that in wild-type cells except that the telomeres in type II survivors are longer and much more heterogeneous. If telomeres of type II survivors consist solely of $C_{1-3}A/TG_{1-3}$ DNA, they should lack recognition sites for most restriction enzymes, even enzymes that cut frequently in yeast DNA. To test this possibility, genomic DNAs from a wild-type strain (Fig. 7, lane wt), a type I survivor (lane I), and 10 independent type II survivors (lanes II) were digested with a mixture of *Alu*I, *Hae*III, *Hin*fI, and *Msp*I. Each of these enzymes recognizes a different 4-bp sequence, and together they are expected to reduce yeast DNA to, on average, 96 bp. There are many sites for these enzymes within Y′, including sites 358 bp downstream of the 5′ end of Y′ and 42 bp upstream of the 3′ end of Y′. The DNA was analyzed by Southern blotting with a $C_{1-3}A/TG_{1-3}$ probe. If many telomeres in type II survivors have long $C_{1-3}A/TG_{1-3}$ tracts, there will be many large fragments that hybridize to a telomeric probe after digestion with the four enzymes.

Since the wild-type strain used for this study had telomeres of ~375 ± 75 bp, digestion with the four enzymes is expected to generate $C_{1-3}A/TG_{1-3}$ hybridizing fragments of ~375 + 42 bp from Y′-bearing telomeres, fragments of ~0.5 kb from tandem Y′ elements, and fragments of up to 1.1 kb from X telomeres. Consistent with this expectation, digestion of DNA from wild-type cells with the four enzymes released $C_{1-3}A/TG_{1-3}$ fragments that were mostly smaller than 1 kb (Fig. 7). DNA from the type I survivor yielded very short fragments, which hybridized to a telomeric probe. In contrast, most fragments containing $C_{1-3}A/TG_{1-3}$ DNA in type II survivors were large, ranging up to 12 kb. The fact that these large fragments hybridized intensely to the $C_{1-3}A/TG_{1-3}$ probe provided additional evidence that they consisted solely of $C_{1-3}A/TG_{1-3}$ DNA. We conclude that most chromosome ends in type II survivors bear very long and variable-length tracts of $C_{1-3}A/TG_{1-3}$ DNA. However, many of the type II survivors also had a subset of telomeres that were as short as those in type I survivors (asterisk in Fig. 7).

Type II survivors are stable over time, but their telomeres continuously shorten. To test whether the telomere structure in type I and type II survivors was stable, three independent type I survivors and 20 independent type II survivors were restreaked 10 times on YEPD plates. DNA was prepared from different restreaks, digested with *Xho*I, and analyzed by Southern blotting with a $C_{1-3}A/TG_{1-3}$ probe (Fig. 8A). Two of the type I survivors maintained a type I telomeric pattern throughout the restreaking period, while the third type I survivor converted to a type II telomeric pattern between the first and fourth restreaks (Fig. 8A, left panel). Concomitant with this switch, the growth rate of this survivor improved and became similar to that of other type II survivors (data not shown). Thus, type I survivors can convert to a type II pattern during outgrowth.

In contrast, all 20 type II survivors retained the *Xho*I pattern of variable-length telomeric fragments characteristic of type II survivors for ~250 cell divisions (~25 cell divisions per restreak) (see Fig. 8A, right panel, for two examples). Although the general pattern of telomeric *Xho*I fragments did not change during subculturing and the average telomere length remained high, individual $C_{1-3}A/TG_{1-3}$-hybridizing *Xho*I fragments in type II survivors appeared to shorten slowly over time (Fig. 8A). This shortening was especially apparent when these blots were reprobed with *his3AI* (Fig. 8B), which detects a single telomere. The rate of telomere shortening in type II survivors was ~3 bp/cell division. The *his3AI* marker at the internal Y′ of the second type II survivor was lost between the fourth and seventh restreaks. Thus, even though the pattern of variable and very long telomeres characteristic of type II survivors was maintained for at least 250 cell divisions, individual type II telomeres continuously shorten and, as inferred from the loss of the *his3AI* tag during outgrowth, engage continuously in gene conversion events with other telomeres.

Normal telomere length regulation is restored in type II survivors when *TLC1* is reintroduced. Since most cells that lack telomerase do not form survivors, generation of survivors might require a second event that activates a telomerase-independent telomere maintenance pathway. If this model were true, reintroduction of telomerase might not be sufficient to restore a wild-type pattern of telomere structure. To determine if the telomere changes that occur in type I and II survivors

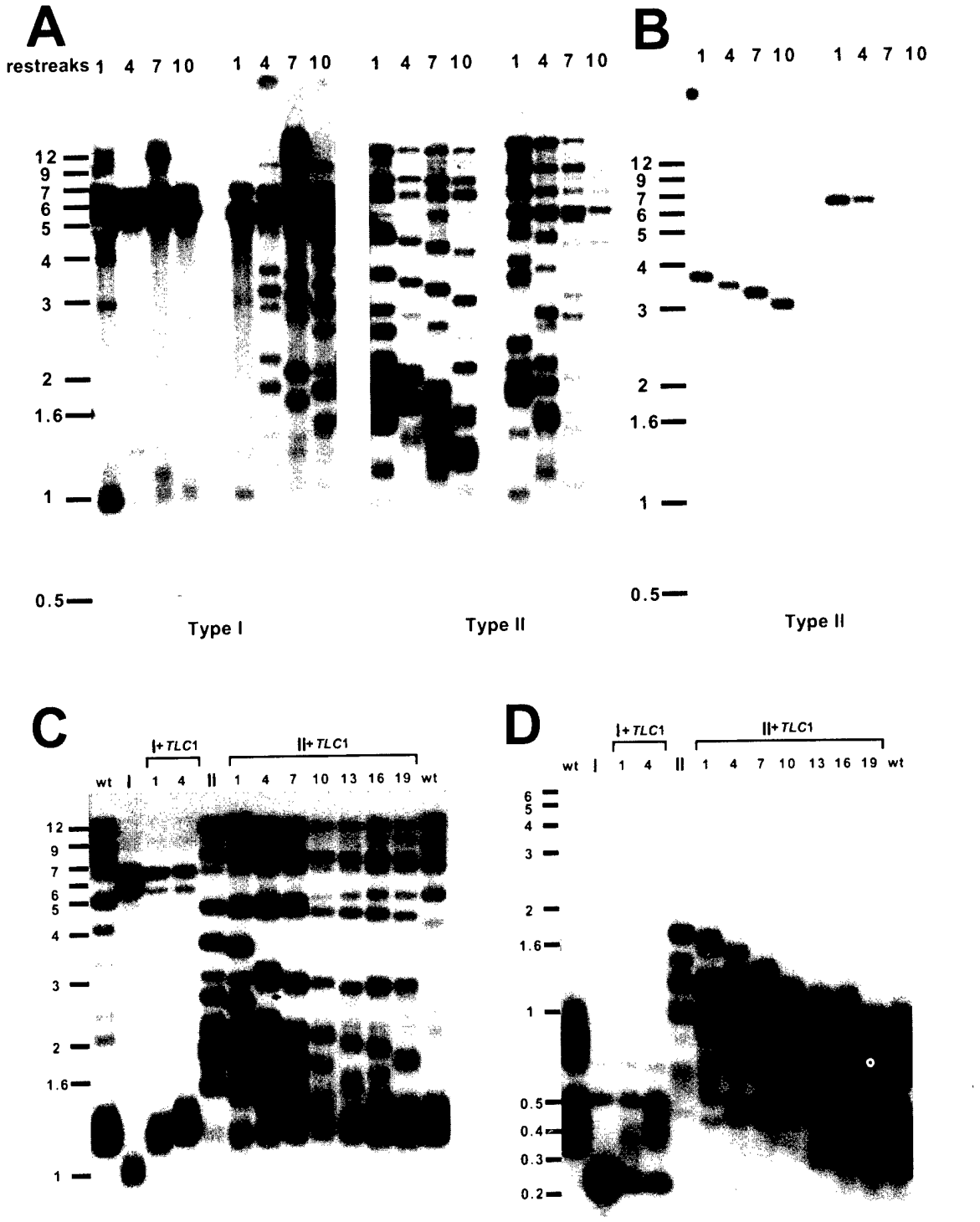

FIG. 8. Telomeric changes in type I and type II survivors during outgrowth and after introduction of telomerase. (A) Stability of telomeric structure in type I and type II survivors. Two type I and two type II survivors were restreaked 10 times on YEPD plates. Genomic DNA from the 1st, 4th, 7th, and 10th restreaks was digested with *Xho*I. Southern blot analysis was performed as in Fig. 1B. (B) Telomeres of type II survivors are subject to continuous shortening and gene conversion. The blot shown on the right of panel A was stripped and rehybridized with a *his3A1* probe. The *his3A1* marker was lost between the fourth and seventh restreaks in the second type II survivor. (C and D) Telomere length reverts to wild type in type I and type II survivors after reintroduction of telomerase. Plasmid pRS317*TLC1* was transformed into both type I and type II survivors. Transformants were restreaked multiple times. Genomic DNA from the 1st and 4th restreaks from a type I survivor and from the 1st, 4th, 7th, 10th, 13th, 16th, and 19th restreaks from a type II survivor was digested with *Xho*I (C) or with a combination of *Alu*I, *Hae*III, *Hin*fI, and *Msp*I (D); fractionated through a 1% agarose gel; and analyzed by Southern blotting with a $C_{1-3}A/TG_{1-3}$ probe. DNAs from the wild type and from the two survivors before introduction of the pRS317*TLC1* plasmid were also analyzed. Similar results were obtained with three additional type II survivors. Size markers are in kilobases. The $C_{1-3}A/TG_{1-3}$ hybridizing fragments in panel C that are >0.5 kb and that are absent in DNA from the type I survivor after telomerase was reintroduced are from X telomeres. Not all type I survivors lacked X telomeres after reintroduction of *TLC1* (data not shown).

were reversible, we introduced a plasmid-borne *TLC1* gene into individual type I and type II survivors. Transformants were restreaked multiple times, and DNA was prepared for Southern analysis after variable number of cell divisions in the presence of telomerase. DNA was digested with both *Xho*I (Fig. 8C) and multiple 4-bp cutters (Fig. 8D). Telomeres of type I survivors reverted to wild-type lengths soon after reintroduction of *TLC1*, whereas it took many generations for type II telomeres to return to wild-type lengths (~475 cell divisions). Similar results were obtained with three other type II survivors. These data suggest that generation of type II survivors does not require mutations of genes involved in telomere length regulation.

DISCUSSION

Previous studies demonstrated that yeast cells lacking telomerase can maintain telomeric DNA by Rad52p-dependent recombination (27). Type I survivors arise by tandem duplication of the subtelomeric Y′ element. Type II survivors were proposed to have deleted Y′ elements but were not characterized in detail (27). To characterize the structure of telomeres in individual type II survivors, we tagged the distal portion of Y′ on individual telomeres with the *his3AI* marker. We showed that cDNA-mediated movement of Y′ to chromosome ends is not a general mechanism for generating type II survivors. We found no evidence for deletion or rearrangement of Y′ elements during the formation of type II survivors (Fig. 3 and 5B). Although the *his3AI* tags remained near a chromosome end (Fig. 5A), the terminal *Xho*I fragments were invariably larger (Fig. 4C and 5A) than in the parental strain. DNA sequencing revealed that the DNA distal of the *his3AI* tag in one type II survivor consisted solely of $C_{1-3}A/TG_{1-3}$ DNA (Fig. 6). Moreover, 10 of 10 type II survivors had heterogeneous-length $C_{1-3}A/TG_{1-3}$ telomeric fragments that were much longer than the $C_{1-3}A/TG_{1-3}$ tracts in wild-type cells (Fig. 7). The simplest interpretation of these data is that telomeres in type II survivors differ from wild-type telomeres by having longer and irregularly extended $C_{1-3}A/TG_{1-3}$ tracts. Since type II survivors required the continuous presence of Rad52p (Fig. 2), the long terminal tracts of $C_{1-3}A/TG_{1-3}$ DNA on type II telomeres are probably maintained by nonreciprocal recombination between two telomeres (38, 46, 47).

In contrast to type I survivors, which often reverted to a type II pattern of telomeric DNA, the type II pattern of telomeres was persistent, being maintained for at least 250 cell generations (Fig. 8A). Despite the stability of the general pattern of telomeric structure in type II survivors, individual telomeres were not static but, rather, steadily shortened (Fig. 8A and B). Moreover, the *his3AI* tags on these telomeres could be lost (Fig. 4C and 7B), duplicated (Fig. 4C to E), or transferred to a different chromosome (Fig. 4D). Since most type II survivors had a growth rate similar to that of wild-type cells, telomere-telomere recombination must be an efficient mechanism for maintaining telomeric DNA. Nonetheless, after reintroduction of telomerase, type II telomeres slowly returned to wild-type lengths (Fig. 8D), suggesting that expression of telomerase in some way suppressed telomere-telomere recombination.

Saccharomyces telomeric $C_{1-3}A/TG_{1-3}$ repeats are associated in vivo with at least eight distinct proteins (4, 16). We propose that as telomeres become critically short, the binding of one or more of these proteins is impaired, an event that could expose the single-stranded 3′ TG_{1-3} tail, freeing it to invade another telomere. Consistent with this possibility, a *tlc1* strain that lacks Rif2p, an in vivo telomere binding protein (4), generates exclusively type II survivors, suggesting that Rif2p normally inhibits telomere-telomere recombination (45a). According to this model, telomere-telomere recombination does not occur once telomerase is reintroduced, because telomerase lengthens critically short telomeres, eliminating the ends that would otherwise initiate recombination. Telomere-telomere recombination in *K. lactis* is also proposed to result from loss of telomere binding proteins (29).

Since all telomeres in a senescing strain are short, a single telomere-telomere recombination event will at most double the size of the recombining telomere. For a telomere to become as long as the typical type II telomere, it must either undergo multiple recombination events with a short telomere or invade a very long telomere. If, as we propose, only critically short telomeres lose telomere binding proteins and initiate telomere-telomere recombination, a short telomere cannot become a long telomere by recombination with another short telomere, because after a single recombination event, it will no longer be critically short. We propose that the rate-limiting step in generating a type II survivor is the creation of one or more telomeres that are long enough to serve as efficient, one-step donors of telomeric DNA. This long telomere might be generated by a series of successive recombination events in which a critically short telomere invades a telomere that was itself lengthened by recombination with another short telomere. Alternatively, formation of the first long telomere might involve a rare replication event, such as repeated replication slippage during gene conversion. If generation of a single long telomere were rare, it would explain why type II survivors are less common than type I. Even after type II telomeres are generated, telomere-telomere recombination must be relatively rare, since individual type II telomeres continuously shortened (Fig. 8B), presumably until they became sufficiently short to initiate recombination.

The pattern of telomere structure in *Saccharomyces* type II survivors is similar to that described in telomerase-minus *K. lactis* cells (29). However, since *K. lactis* survivors go through repeated rounds of telomere elongation, telomere shortening, and senescence (29), their growth characteristics are more similar to those of type I than type II *Saccharomyces* survivors, perhaps because telomere-telomere recombination is even rarer in *K. lactis* than in *Saccharomyces*. The structure of telomeres in type II survivors in *Saccharomyces* was also similar to that described in human cells that maintain their telomeres by the telomerase-independent alternative lengthening of telomeres (ALT) pathway (7, 8). These cells also have very long and heterogeneous-length telomeres. Moreover, individual telomeres in cells maintaining telomeres by the ALT pathway are seen to both gradually shorten and undergo rapid, one-step elongation (32).

In summary, previous work showed that linear chromosomes in *Saccharomyces* could be maintained either by telomerase or by recombination-driven amplification of subtelomeric Y′ DNA (27). Here we show that there is yet a third mechanism that can maintain the ends of yeast chromosomes, telomere-telomere recombination. Although this pathway appears to be as efficient as telomerase in maintaining linear chromosomes, its occurrence is suppressed in telomerase-proficient cells.

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Pif1p Helicase, a Catalytic Inhibitor of Telomerase in Yeast

J-Q. Zhou, E.K. Monson, [+], S-C. Teng,[+], V.P. Schulz,[1+], and V.A. Zakian*

Department of Molecular Biology, Princeton University, Princeton NJ 08544-1014. [1]Current address: Genaissance Pharmaceuticals, Five Science Park, New Haven, CT 06511

*To whom correspondence should be addressed: vzakian@molbio.princeton.edu

[+] These authors contributed equally to this work

Abstract

Mutations in the yeast *Saccharomyces cerevisiae PIF1* gene, which encodes a 5' to 3' DNA helicase, cause telomere lengthening and a large increase in the rate of forming new telomeres. Here we show that Pif1p acts by inhibiting telomerase, not telomere-telomere recombination, and this inhibition requires the helicase activity of Pif1p. Over-expression of enzymatically active Pif1p causes telomere shortening. Thus, Pif1p is a catalytic inhibitor of telomerase-mediated telomere lengthening. As Pif1p is associated with telomeric DNA *in vivo*, its effects on telomeres are likely direct. Pif1p-like helicases are found in diverse organisms, including humans. We propose that Pif1p-mediated inhibition of telomerase promotes genetic stability by suppressing telomerase-mediated healing of double strand breaks.

PIF1 is a non-essential *Saccharomyces* gene that encodes a 5' to 3' DNA helicase (*1*). Mutations in *PIF1* affect telomeres in three ways: *pif1* telomeres are longer than wild type telomeres; healing of double strand breaks by telomere addition occurs much more often in *pif1* than wild type cells; and *pif1* but not wild type cells add telomeric DNA to sites that have very little resemblance to telomeric DNA (*2*). These data suggest that Pif1p is an inhibitor of telomere lengthening. Pif1p also affects mitochondrial (*1*) and ribosomal DNA (*3*).

There are two mechanisms that can lengthen the ~300 bp tracts of yeast telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA, telomerase (*4*) and telomere-telomere recombination (*5*). In the absence of genes required for telomerase, such as *TLC1*, which encodes telomerase RNA (*6*) and *EST1*, which encodes a telomerase RNA binding protein (*7*), telomeric DNA gets shorter and shorter, the cultures senesce, and most cells eventually die. Lengthening of telomeres by recombination requires the continued presence of Rad52p (*5*). If Pif1p inhibits telomere-telomere recombination, telomere lengthening will not occur in a *pif1 rad52* strain and a *pif1 tlc1* (or *est1*) strain might not senesce or would senesce more slowly due to activation of the recombinational pathway for telomere maintenance. If Pif1p inhibits telomerase, telomere lengthening would not occur in *pif1 tlc1* or *pif1 est1* strains. To distinguish between these possibilities, we constructed singly and doubly mutant strains of the appropriate genotypes and examined telomere lengths (*8*). Because telomeres were at least as long in a *pif1 rad52* as a *pif1* strain (Fig. 1A), the effects of Pif1p did not require Rad52p. In contrast, telomere lengthening did not occur in either a *pif1 tlc1* (Fig. 1B) or a *pif1 est1* strain (data not shown). In addition, lack of Pif1p did not bypass or even delay the senescence phenotype of cells lacking telomerase (Fig. 1C). Thus, Pif1p inhibits a telomerase-dependent pathway of telomere lengthening.

To determine if the helicase function of Pif1p is required to inhibit telomere lengthening, we used site directed mutagenesis to modify the invariant lysine in the ATP binding domain to either

alanine (K264A) or arginine (K264R) (*9*) as this residue is essential for the activity of other helicases (*10*). Both wild type and the K264A mutant version of Pif1p were expressed in Sf9 insect cells infected with recombinant baculovirus, purified to near homogeneity (Fig. 2A), and their activities assayed *in vitro*. Whereas wild type Pif1p catalyzed unwinding of a 17 base ^{32}P end labeled oligonucleotide annealed to a M13 single strand circle, the K264A allele had no helicase activity in this assay (Fig. 2B).

To determine the phenotype of cells that lacked Pif1p helicase activity, strains with only the K264A or K264R allele were constructed (*11*). DNA was prepared from cells carrying these mutant alleles as well as from wild type, *pif1Δ*, *pif1-m1*, or *pif1-m2* strains and examined by Southern analysis (Fig. 2C). The *pif1-m1* and *pif1-m2* alleles are point mutations in, respectively, the first or second AUG of the *PIF1* open reading frame: *pif1-m1* cells have mutant mitochondrial function but wild type telomeres whereas *pif1-m2* cells have wild type mitochondria but mutant telomeres *(2)*. As expected, *pif1Δ* (lane 2) and *pif1-m2* (lane 4) cells had long telomeres whereas *pif1-m1* cells (lane 3) had telomeres of wild type length (lanes 1 & 9). The strains with the K264A and K264R alleles also had long telomeres (lanes 5 & 6). That this telomere lengthening was due to the point mutations in the ATP binding pocket was demonstrated by restoration of wild type telomere length in K264A and K264R cells carrying a plasmid borne copy of wild type *PIF1* (lanes 7 & 8).

Western analysis established that cells carrying the K264A and K264R alleles produced stable Pif1p (Fig. 2D). Wild type cells contained two similarly sized proteins of ~94 kDa, the expected size for Pif1p (lane 1). The mitochondrially defective *pif1-m1* strain expressed only the longer form of Pif1p (lane 3) whereas the telomere impaired *pif1-m2* strain expressed only the faster migrating species (lane 4). These data provide direct evidence for the hypothesis (2) that Pif1p is targeted to different sub-cellular compartments by making two forms of the protein, one localized to mitochondria and the other, whose translation begins at the second AUG in the open reading frame, destined for the nucleus. (The

mitochondrial Pif1p was shorter than nuclear Pif1p due to proteolysis during import into mitochondria). As the K264A and K264R alleles produced close to wild type levels of the nuclear form of Pif1p (lanes 5 & 6), their effects on telomere length must be due to loss of Pif1p ATPase/helicase activity.

If Pif1p is an inhibitor of telomerase, over-expression of Pif1p might result in telomere shortening. To test this possibility, *PIF1* was cloned into the multi-copy YEpFAT7 plasmid (*12*). *PIF1* cells carrying YEpFAT7-*PIF1* had telomeres that were ~80 bp shorter than cells carrying YEpFAT7 (Fig. 2E). In contrast, wild type cells carrying YEpFAT7-K264A *PIF1* did not show telomere shortening. The fact that over-expression of wild type but not helicase deficient Pif1p caused telomere shortening is consistent with the idea that Pif1p acts enzymatically to inhibit telomerase.

Mutations in certain essential replication proteins also cause telomere lengthening when cells are grown at semi-permissive temperatures for loss of function alleles (*13*). Analysis by fluorescence activated cell sorting (FACS) shows that the replication mutants with lengthened telomeres had an abnormally large number of S phase cells. As telomeric DNA is replicated at the end of S phase (*14*), changes in telomere length in these mutants might be linked to their genome wide slowing of DNA replication. In contrast, the FACS profile of *pif1* cells revealed that they had a similar fraction of S phase cells as the isogenic wild type strain (Fig. 3A). In addition, cells lacking Pif1p have wild type rates of chromosome loss and mitotic recombination *(2)*, whereas mutants in general replication proteins increase both (*15*). These data suggest that Pif1p has a direct effect on telomere replication.

If Pif1p acts directly to inhibit telomere replication, it should associate physically with telomeric DNA. To assess this possibility, chromatin was cross-linked *in vivo* with formaldehyde, sheared to ~1000 bps and then precipitated with either protein A-purified pre-immune antibodies (rabbit IgG) or affinity purified anti-Pif1p antibodies (α-Pif1p) (Fig. 3B). As a positive control, chromatin was also precipitated with an anti-Rap1p serum (α-Rap1p): Rap1p, the major structural protein at yeast telomeres

is constitutively bound to telomeric DNA (*16*). The cross-links in the immuno-precipitate were reversed and the DNA in the immuno-precipitate was PCR amplified using primers that amplified a 233 bp portion of the sub-telomeric Y' element that lies 30 bps upstream of the start of the telomeric repeats or, as a negative control, primers for a 131 bp fragment of the *ACT1* gene. Both the anti-Rap1p and anti-Pif1p serum specifically precipitated telomeric DNA. Two fold serial dilutions of immuno-precipitates revealed that telomeric DNA was enriched 5.0 ± 2.0 fold (mean ± SD) in the anti-Pif1p precipitate compared to its presence when pre-immune antibodies were used or when the anti-Pif1p antibodies were used to precipitate chromatin from *pif1Δ* cells. As Pif1p was not telomere associated in the absence of cross-linking (X-link -), its association must have occurred *in vivo*.

When the sequence of the 859 amino acid Pif1p was compared to the translated DNA data base, several highly similar genes were detected including a second S. *cerevisiae* gene called *RRM3* (Fig. 4A). None of the other 132 yeast ORFs with helicase motifs (*17*) had detectable similarity to Pif1p by the criterion of a blast search (*18*). This search also identified Pif1p-like proteins in *C. maltosa*, *C. elegans*, and *D. melanogaster* (Fig. 4A). We isolated *PIF1*-like genes from both *S. pombe* (*rph1*$^+$, *RRM3/PIF1* homologue) and *H. sapiens* (hPif1p, human Pif1p) (*19*). Thus, Pif1p is the prototype member of a helicase sub-family, conserved from yeasts to humans. Pif1 sub-family members encode proteins with 30 to 50% identity in all pair wise combinations over a region of over 300 amino acids (Fig. 4A). This degree of relatedness is similar to or greater than that seen within other helicase sub-families (*20*). As all of the Pif1p-like proteins had very high identity to Pif1p, a known DNA helicase, within each of the helicase motif regions (*21*), the other members of the Pif1p subfamily are also likely to be helicases. Like Pif1p, the *Saccharomyces* Rrm3p and the *S. pombe* Rph1p also affect telomeric DNA (*18*).

This paper demonstrates that the *Saccharomyces* Pif1p inhibits telomerase lengthening of telomeric DNA (Fig. 1). Telomere length was inversely proportional to the amount of Pif1p in cells.

Loss of Pif1p led to telomere lengthening (Fig. 2C) and over-expression of Pif1p caused telomere shortening (Fig. 2E). The catalytic activity of Pif1p was required for both of these effects (Fig. 2C, E). Although helicases are required for transcription, RNA processing and translation, as well as for DNA replication, the association of Pif1p with telomeric DNA *in vivo* (Fig. 3B) argues strongly that its effects on telomeres are direct.

How might a 5' to 3' DNA helicase counter telomerase activity? Because yeast chromosomes have 3'single-strand tails (*22*), Pif1p does not have the right polarity to unwind chromosomes from their ends (Fig. 4B, left). However, Pif1p could dissociate the last Okazaki fragment to generate a long single-strand $TG_{1\text{-}3}$ tail (Fig. 4B, middle). Although a priori, G-tails seem more likely to stimulate than inhibit telomerase, $TG_{1\text{-}3}$ tails might fold into a structure that prevents telomerase lengthening (*23*, *24*). Alternatively, the helicase activity of Pif1p might dissociate telomeric DNA from telomerase RNA (Fig. 4B, right). If Pif1p preferentially dissociates RNA-DNA hybrids held together by a very small number of base pairs, it would explain the reduced specificity of telomere addition seen in the absence of Pif1p (*2*).

Is there an advantage to inhibiting telomerase? Cells lacking the nuclear form of Pif1p had no cell cycle defect (Fig. 3A) and wild type chromosome stability (*2*). Thus, Pif1p is not important in the normal mitotic cell cycle. However, Pif1p might be critical after DNA damage. When a yeast chromosome loses a telomere, the broken chromosome is either lost or a new telomere is gained by homologous recombination (*25*). Although wild type cells very rarely add telomeres *de novo* to broken chromosomes (*2*, *26*), the rate of *de novo* telomere addition in *pif1* cells is elevated as much as 600 fold (*2*). Adding a telomere to a double strand break results in aneuploidy for sequences distal to the site of telomere addition. By inhibiting such events, Pif1p could promote genetic stability.

Figure legends.

Figure 1: Pif1p inhibits telomerase, not telomere-telomere recombination.

A. DNA was prepared from three independent transformants from otherwise isogenic strains of the indicated genotypes. The DNA was digested with *Xho*I and analyzed by Southern hybridization using a $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric probe here and in panel B. The *pif1-m2* allele, which affects telomeric but not mitochondrial DNA *(2)* was used here and in panels B & C. **B.** A diploid strain heterozygous at both *TLC1* and *PIF1* was sporulated, tetrads were dissected, and the genotype of the spore products was determined. DNA was isolated from independent spores with the indicated genotypes ~30 cell divisions after sporulation. **C.** Individual spores from tetrads obtained as in B were streaked on rich medium and grown to single colonies (~25 cell divisions). Individual colonies were resteaked repeatedly. The third and fourth restreaks after sporulation are shown for the four spore products from one of nine tetrads examined.

Figure 2: Pif1p helicase activity is essential for inhibition of telomere lengthening.

A. Recombinant wild-type Pif1p and mutant Pif1p-K264A were purified from Sf9 insect cells infected with recombinant virus, and analyzed with silver stained SDS-PAGE (left panel) or western blotting with affinity purified antibodies against Pif1p (right panel). **B.** Helicase activity assays were carried out using 1 μM of the partial duplex DNA substrate (a 5' ^{32}P end labeled 17-nucleotide oligomer annealed to the single stranded M13mp7 DNA) and 50 ng of purified recombinant protein (panel A). Reactions were incubated at 37^{O}C for 30 min and analyzed by 10% PAGE and autoradiography. **C.** Genomic DNA from wild type and mutant strains was analyzed as in Fig. 1A. The lanes contain DNA from otherwise isogenic wild-type (lane 1), *pif1Δ* (lane 2), *pif1-m1* (lane 3), *pif1-m2* (lane 4), *pif1-K264A* (lane 5), *pif1-K264R* (lane 6), *pif1-*

K264A carrying pVS102, a centromere plasmid bearing wt *PIF1* (lane 7), *pif1-K264R* carrying pVS102 (lane 8), and wild-type (lane 9). **D.** Western analysis on proteins isolated from the mutant and wild type cells whose DNA was examined in panel C using affinity-purified anti Pif1p serum. Lanes are the same as in panel C. **E.** Genomic DNA was isolated from three independent cultures of *PIF1* cells carrying either the multi-copy vector YEpFAT7, YEpFAT7 containing the *PIF1* gene, or YEpFAT7-containing the *PIF1*-K264A mutant gene and analyzed by Southern blotting as in Fig. 1A.

Figure 3. Pif1p affects telomeric DNA directly.

A. Log phase *pif1-m2* and otherwise isogenic wild type cells, were grown in rich medium, fixed, stained with propidium iodide, and analyzed by fluorescence activated cell sorting (FACS). **B.** Chromatin was prepared from otherwise isogenic wt or *pif1Δ* cells that had been cross-linked (X-link +) or not (X-link -) with formaldehyde *in vivo*. Immuno-precipitation was carried out as described in *(3)* using either protein A-purified pre-immune IgG (rabbit IgG), a polyclonal Rap1p antiserum (α-Rap1p) (*16*), or affinity purified anti-Pif1p polyclonal antibodies (α-Pif1p). The DNA in the immuno-precipitate was PCR amplified for 28 cycles using telomeric primers or for 32 cycles using *ACT1* primers, separated in an agarose gel, and visualized by staining with ethidium bromide. PCR amplification of the input DNA with telomeric primers is also shown. Although Pif1p association with telomeric DNA did not occur in the absence of cross-linking, the amount of telomeric DNA precipitated with anti-Rap1p was not eliminated in non-cross linked cells.

Figure 4: *PIF1* is the prototype of a sub-family of putative helicases.

A. The predicted sequences of the *PIF1*-like proteins were compared using the TBLASTN 2.06 program (*27*). The top line for each pair shows the expectation value, a measure of the probability that the match occurred by chance. The number of amino acids of homology shared between the two proteins and percentage identity within the helicase region is shown below. These numbers were obtained by aligning the helicase region using the MacVector 6.0 (Oxford Molecular) implementation of the ClustalW program (*28*). **B.** Models for Pif1p inhibition of telomerase lengthening. The left telomere of a single chromosome is shown. The solid circle is Pif1p. Left: a 3' to 5' helicase could unwind chromosomes from their ends to create a substrate for a nuclease that inhibits telomerase by destroying its substrate. As Pif1p is a 5' to 3' helicase, this model can not explain the effects of Pif1p. Middle: Pif1p might dissociate the last Okazaki fragment to generate a ~100 base $TG_{1\text{-}3}$ tail. This single strand $TG_{1\text{-}3}$ tail could inhibit telomerase either by forming a higher order structure such as a G-quartet (*23*) or t-loop (*24*) or by serving as a substrate for an exo-nuclease. The open rectangle represents the 8-12 base RNA that primes Okazaki fragments. Right: Pif1p might inhibit telomerase directly by promoting dissociation of the telomerase RNA-telomeric DNA hybrid that is an intermediate in telomere replication. The structure base paired to the 3' single strand tail is telomerase RNA.

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8. *S. cerevisiae* strains isogenic to VPS106 were used *(2)* (Fig. 1). *RAD52* was inactivated by transforming cells with a fragment containing *LEU2* inserted at the unique *Bgl*II site within the coding region of *RAD52*. The *TLC1* gene was inactivated by transforming diploid VPS107 *PIF1/pif1-m2* cells with *Xho*I digested pBlue61::LEU2 which deletes 693 bps from the *TLC1* coding region *(6)*. The resulting diploid strain,VPS107*PIF1/pif1-m2 TLC1/tlc1::LEU2*, was sporulated, and tetrads were dissected. Individual spore products from nine tetrads were streaked four times on rich medium and examined at each streak for growth rate and viable colonies. DNA for analysis of telomere length was prepared ~30 cell divisions after sporulation and ~5 cell divisions after each restreak.

9. Mutagenesis of lysine 264 in *PIF1* to alanine (K264A) or arginine (*K264R*) was done using the QuickChange™ Site-directed Mutagenesis Kit (Stratagene) on the 488 bp *Afl*II-*Pfl*MI *PIF1* fragment which was sequenced after mutagenesis to ensure that it contained only the desired mutation. The mutagenized fragment was inserted into pVS102, which contains a genomic *Sac*I-*Apa*I *PIF1* fragment, replacing the wild type portion of the gene. The pVS102-mutant or wild type *PIF1* plasmids were *Sac*I -*Cla*I digested and the fragment containing *PIF1* inserted at the *Bam*HI site in the pFastBac1 vector (GIBCO BRL). For the purification of recombinant proteins, 1 liter of Sf9 cells at a density of 1.5 x 10^6 /ml were infected at a multiplicity of 10, and harvested at 60 hr post-infection. The recombinant proteins in the cytoplasmic fraction were followed by Western blotting, using a Pif1p antiserum *(3)* and purified with chromatography on phosphor-cellulose, Q sepharose and heparin agarose.

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11. Experiments to assess the effects of eliminating the helicase activity of Pif1p were carried out in strain YPH499 (*29*). The *pif1Δ* allele deleted 1465 bps, extending from the first initiation codon of *PIF1* through amino acid 488. The K264A and K264R *PIF1* alleles were introduced by intergrative transformation in such a way as to replace the endogenous *PIF1*. The presence of point mutations was confirmed by DNA sequencing on PCR amplified DNA. Pif1p was overproduced in YPH499 cells by inserting a *Sac*I-*Apa*I *PIF1* fragment which contained the *PIF1* ORF as well as 1291 bps 5' and 2788 bps 3' of the ORF into *Sma*I digested YEpFAT7 to yield pVS41. Chromatin immuno-precipitations were done in the YPH499 background.

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19. The *S. pombe rph1*$^+$ was isolated using PCR primers that were based on conserved amino acid residues present in both *PIF1* and the *C. maltosa* Pif1p-like protein (accession number AF074944). By comparing the Pif1p sequence with the translated database of expressed sequence tags, we found one human gene (accession number AA464521) with low levels of similarity (expectation value of 0.015). We extended this sequence 3' to the poly A tail by further searching of the human gene against the EST database (unigene number Hs.112160). The raw sequence was corrected by inspection of the automated sequencing traces, and when this sequence was compared to the protein database, high

levels of similarity to the *PIF1* sub-family were found. We extended the sequence deduced from the database to 374 amino acids Accession number (AF108138) using rapid amplification of cDNA ends. We were unable to obtain the sequence of the amino terminal portion of hPif1 because after 374 amino acids its cDNA was fused to an Alu element as a result of template switching during cDNA synthesis.

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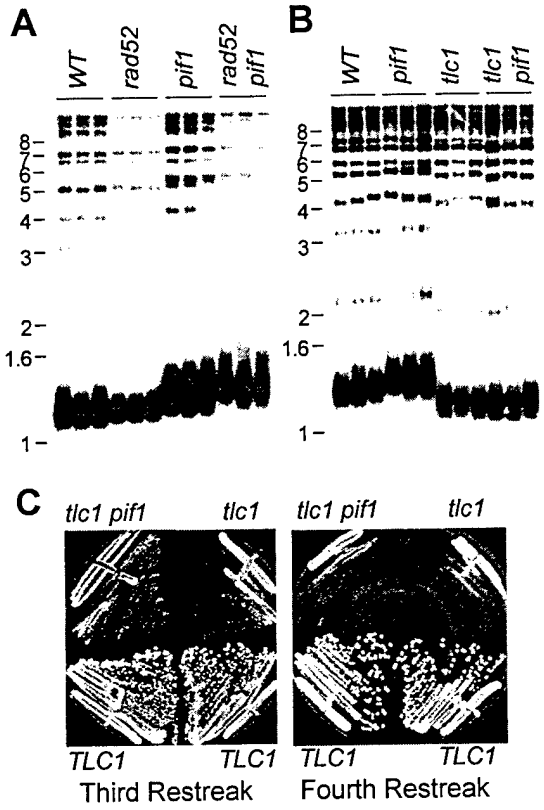
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1.6
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tlc1
tlc1 pif1
8
7
6
5
4
3
2
1.6
1
C
tlc1 pif1
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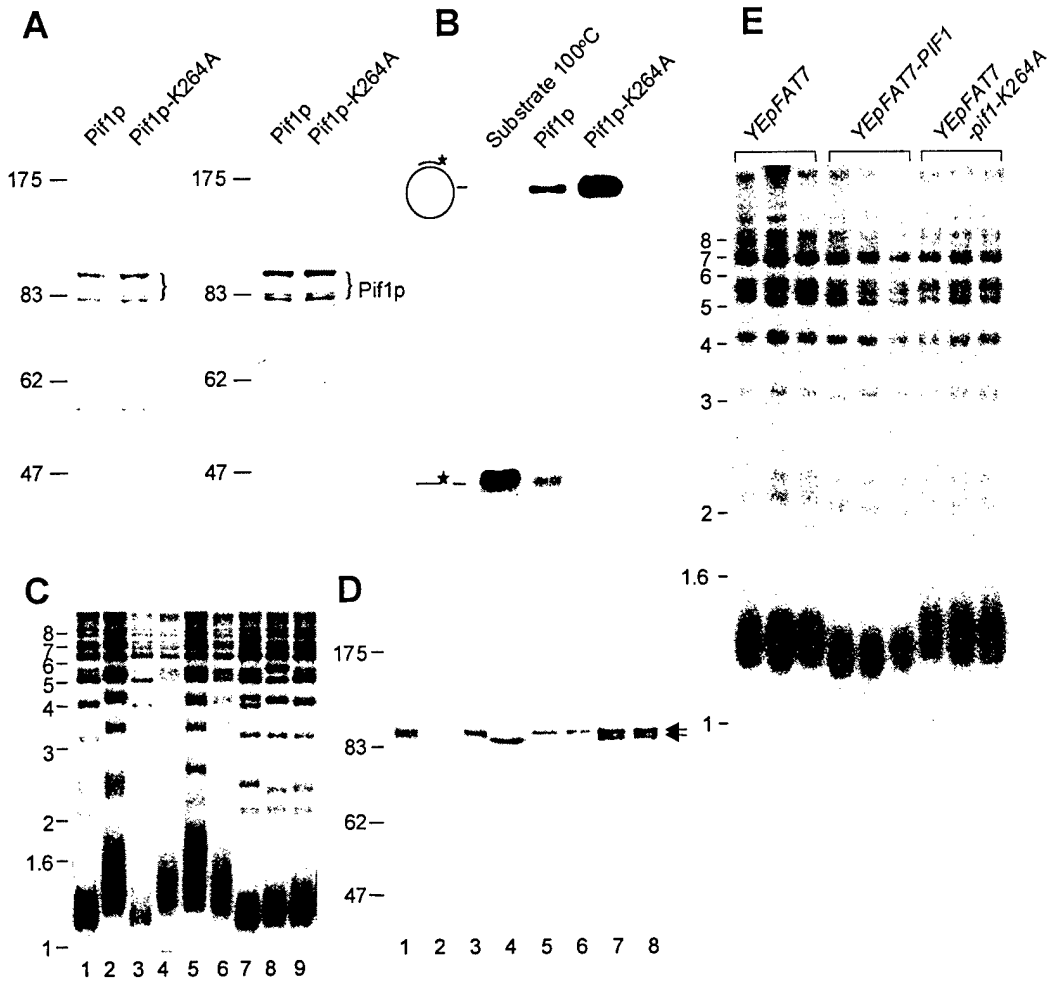
TLC1
TLC1
TLC1
Third Restreak
Fourth Restreak

Fig. 1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C
8
7
6
5
4
3
2
1.6
1
1 2 3 4 5 6 7 8 9
D
175
83
62
47
1 2 3 4 5 6 7 8
E
YEpFAT7
YEpFAT7-PIF1
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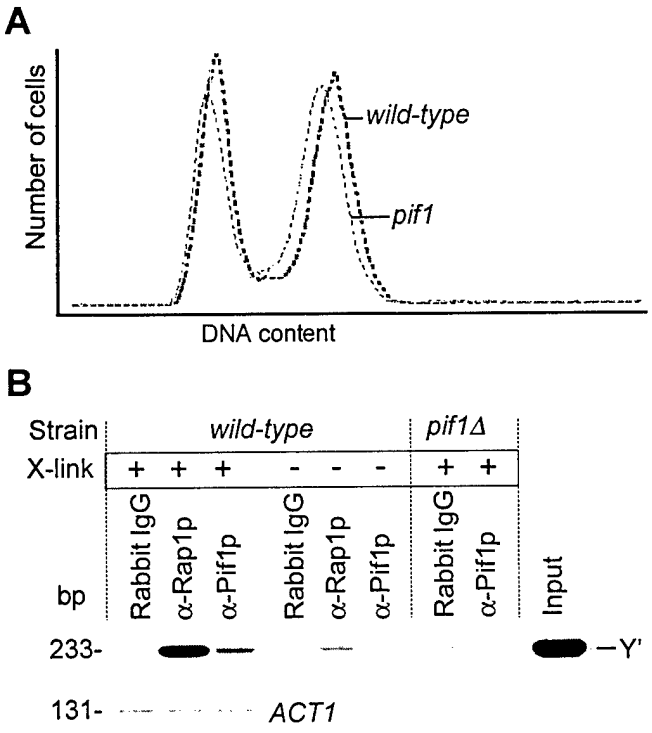
8
7
6
5
4
3
2
1.6
1

Fig. 2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DNA content
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Strain
wild-type
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α-Pif1p
Rabbit IgG
α-Rap1p
α-Pif1p
Rabbit IgG
α-Pif1p
Input
bp
233-
131-
Y'
ACT1

Fig. 3

A

	PIF1	*RRM3*	*rph1*	*C. maltosa*	*C. elegans*	*D. melanogaster*
RRM3	10^{-97} 485 aa, 38%					
rph1	10^{-116} 488 aa, 41%	10^{-104} 449 aa, 43%				
C. maltosa	10^{-55} 371 aa, 34%	10^{-63} 342 aa, 38%	10^{-55} 339 aa, 35%			
C. elegans	10^{-57} 484 aa, 33%	10^{-61} 443 aa, 35%	10^{-77} 428 aa, 36%	10^{-25} 337 aa, 28%		
D. melanogaster	10^{-48} 323 aa, 38%	10^{-47} 316 aa, 39%	10^{-67} 315 aa, 44%	10^{-19} 184 aa, 35%	10^{-98} 616 aa, 37%	
H. sapiens	10^{-41} 438 aa, 32%	10^{-41} 395 aa, 32%	10^{-59} 377 aa, 36%	10^{-26} 344 aa, 33%	10^{-75} 324 aa, 43%	10^{-96} 352 aa, 50%

B

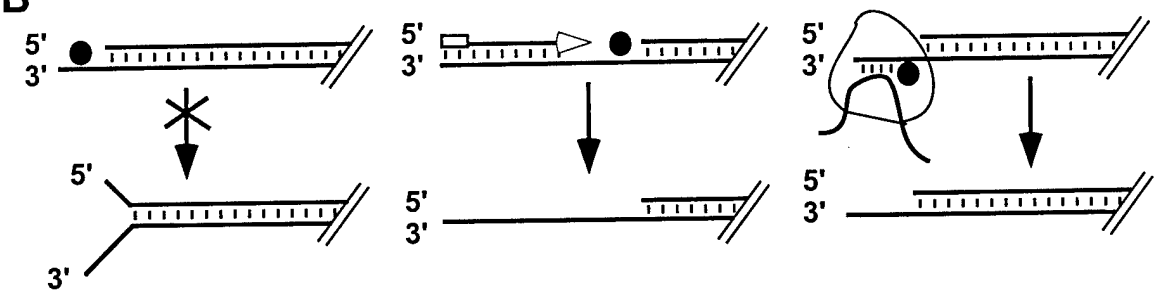

Fig. 4

Science on Line Material for Zhou, Monson, Teng, Schulz, and Zakian, Pif1p helicase, a catalytic inhibitor of telomerase:

The portion of Pif1p and the Pif1p-like proteins containing the seven helicase motifs (1) were aligned using the ClustalW program (2). Black shaded residues are 100% conserved, grey shaded white residues are ≥80% conserved, and grey shaded black residues are ≥60% conserved. Dashes indicate gaps in the sequence or in the case of the amino terminus of hPif1, the sequence has not been determined. Pif1p is a member of helicase superfamily1 (SF1): the SF1 consensus sequence for the seven motifs (HEL cons.)(1) and the consensus for the Pif1 subfamily (*PIF1* cons.) are presented. Symbols: +, hydrophobic residue; o, hydrophilic residue; x, any residue. Small letters indicate that the indicated amino acid is found in ≥80% of the proteins being compared. An asterisk marks the lysine residue in the ATP binding motif I that was mutated to either alanine or arginine. The numbers to the right of the gene name indicates the number of amino acids to the amino terminus of the proteins. Sizes in amino acids of full length proteins are 859 (Pif1p), 723 (Rrm3p), 805 (*S. pombe* Rph1p), 677 (*C. elegans* protein), and 663 (*D. melanogaster* protein). The size of the human protein is not known.

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